

**TREATMENT OF VASCULITIDES USING TNF $\alpha$  INHIBITORS**

**RELATED APPLICATIONS**

This application claims priority to prior filed U.S. Provisional Application Serial  
5 No. 60/397,275, filed July 19, 2002. This application also claims priority to prior filed  
to U.S. Provisional Application Serial No. 60/411,081, filed September 16, 2002, and  
prior-filed U.S. Provisional Application Serial No. 60/417490, filed October 10, 2002.  
This application also claims priority to prior filed to U.S. Provisional Application Serial  
No. 60/455777, filed March 18, 2003. In addition, this application is related to U.S.  
10 Patent Nos. 6,090,382, 6,258,562, and 6,509,015. This application is also related to U.S.  
Patent Application Serial No. 10/302356, filed November 22, 2002; U.S. Patent  
Application Serial No. 09/801,185, filed March 7, 2001; U.S. Patent Application Serial  
No. 10/163657, filed June 2, 2002; and U.S. Patent Application Serial No. 10/133715,  
filed April 26, 2002.

15 This application is related to U.S. utility applications (Attorney Docket No. BPI-  
187) entitled "Treatment of TNF $\alpha$ -Related Disorders Using TNF $\alpha$  Inhibitors," (Attorney  
Docket No. BPI-188) entitled "Treatment of Spondyloarthropathies Using TNF $\alpha$   
Inhibitors," (Attorney Docket No. BPI-189) entitled "Treatment of Pulmonary Disorders  
Using TNF $\alpha$  Inhibitors," (Attorney Docket No. BPI-190) entitled "Treatment of  
20 Coronary Disorders Using TNF $\alpha$  Inhibitors," (Attorney Docket No. BPI-191) entitled  
"Treatment of Metabolic Disorders Using TNF $\alpha$  Inhibitors," (Attorney Docket No. BPI-  
192) entitled "Treatment of Anemia Using TNF $\alpha$  Inhibitors," (Attorney Docket No.  
BPI-193) entitled "Treatment of Pain Using TNF $\alpha$  Inhibitors," (Attorney Docket No.  
BPI-194) entitled "Treatment of Hepatic Disorders Using TNF $\alpha$  Inhibitors," (Attorney  
25 Docket No. BPI-195) entitled "Treatment of Skin and Nail Disorders Using TNF $\alpha$   
Inhibitors," (Attorney Docket No. BPI-196) entitled "Treatment of Vasculitides Using  
TNF $\alpha$  Inhibitors," (Attorney Docket No. BPI-197) entitled "Treatment of TNF $\alpha$ -  
Related Disorders Using TNF $\alpha$  Inhibitors," and PCT application (Attorney Docket No.  
BPI-187PC) entitled "Treatment of TNF $\alpha$ -Related Disorders," all of which are filed on  
30 even date herewith. The entire contents of each of the above-mentioned patents and  
patent applications are hereby incorporated herein by reference.

## **BACKGROUND OF THE INVENTION**

Vasculitis is a group of diseases that involve inflammation in blood vessels. The prevalence of certain specific types of vasculitis has been well studied in the United States. About 100,000 Americans per year are hospitalized for care of vasculitis. Children and adults, males and females, and individuals of any ethnic background may be affected. Systemic vasculitis is often life threatening and likely to produce disability or death. For example, in the case of Wegener's granulomatosis, approximately 1500 patients are hospitalized for this illness in the US every year. Eleven percent die in the course of hospitalization, 31% become totally disabled in performing their usual occupation and 20% become partially disabled over 5 years from the time of disease onset (Hoffman, G.S., *et al.* (1998) *Arthritis Rheum.* 41:2257; Cotch MF. (2000) *Curr. Opin. Rheumatol.* 12:20-3). Because these and related disorders are uncommon, medical expertise to provide care is limited, care may be compromised, and unnecessary morbidity and mortality often occur. Even in the best of hands, treatment in many cases of vasculitis is not curative and toxicity from typical therapy (*e.g.*, high-dose corticosteroids in combination with cytotoxic drugs (cyclophosphamide, azathioprine, methotrexate) is universal. This in turn leads to permanent sequelae in many patients.

Cytokines, such as TNF $\alpha$  (also referred to as TNF), are produced by a variety of cells, and have been identified as mediators of inflammatory processes, including vasculitides. Cytokines regulate the intensity and duration of the inflammatory response which occurs as the result of an injury, disease, or infection. Tumor necrosis factor (TNF) is a cell associated cytokine which is produced by numerous cell types, including monocytes and macrophages, that was originally identified based on its capacity to induce the necrosis of certain mouse tumors (see *e.g.*, Old, L. (1985) *Science* 230:630-632). TNF has been shown to be a primary mediator in humans and in animals, of inflammation, fever, and acute phase responses, similar to those observed during acute infection and shock.

## **SUMMARY OF THE INVENTION**

The present invention provides methods and compositions for the treatment or prevention of vasculitides, including the treatment or prevention of Behcet's disease.

Excessive or unregulated TNF $\alpha$  production has been implicated in mediating a number of diseases, including vasculitis (Deguchi *et al.* (1989) *Lancet*. 2:745). Patients suffering from vasculitis, have elevated levels of TNF $\alpha$  expression (Deguchi *et al.* (1990) *Clin Exp Immunol.* 81:311).

5           The invention describes a method of treating a subject suffering from vasculitis comprising administering a therapeutically effective amount of a TNF $\alpha$  antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF $\alpha$  with a  $K_d$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, both determined by surface plasmon resonance, and neutralizes human TNF $\alpha$   
10   cytotoxicity in a standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-7}$  M or less, such that the vasculitis is treated.

          The invention also provides a method of treating a subject suffering from vasculitis comprising administering a therapeutically effective amount a TNF $\alpha$  antibody, or an antigen-binding fragment thereof, wherein the antibody dissociates from human  
15   TNF $\alpha$  with a  $K_{off}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain  
20   comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12, such that the vasculitis is treated.

          The invention also includes a method of treating a subject suffering from  
25   vasculitis comprising administering a therapeutically effective amount a TNF $\alpha$  antibody, or an antigen-binding fragment thereof, with a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2, such that the vasculitis is treated.

30           In one embodiment of the invention, the antibody, or antigen-binding fragment thereof, is D2E7, also referred to as HUMIRA<sup>®</sup> (adalimumab).

In one embodiment of the invention, the vasculitis is a large vessel disease. In another embodiment, the vasculitis is a medium vessel disease. An example of a medium vessel disease included in the treatment methods of the invention is Kawasaki's Disease. In another embodiment, the vasculitis is a small vessel disease. In still another  
5 embodiment, the small vessel disease is Behcet's Syndrome.

In one embodiment, the invention provides a method of treating vasculitis, wherein the vasculitis is selected from the group consisting of giant cell arteritis, temporal arteritis, polymyalgia rheumatica, Takayasu's disease, polyarteritis nodosa, Kawasaki's disease, Behcet's Syndrome, Wegener's granulomatosis, and Churg-Strauss  
10 syndrome.

The invention also provides a method of treating Behcet's disease in a subject comprising administering a therapeutically effective amount of a  $\text{TNF}\alpha$  antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human  $\text{TNF}\alpha$  with a  $K_d$  of  $1 \times 10^{-8}$  M or less and a  $K_{\text{off}}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or  
15 less, both determined by surface plasmon resonance, and neutralizes human  $\text{TNF}\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  $\text{IC}_{50}$  of  $1 \times 10^{-7}$  M or less, such that said Behcet's disease is treated or prevented.

In one embodiment, the invention describes a method of treating or preventing Behcet's disease in a subject comprising administering a therapeutically effective  
20 amount a  $\text{TNF}\alpha$  antibody, or an antigen-binding fragment thereof, wherein the antibody has the following characteristics: dissociates from human  $\text{TNF}\alpha$  with a  $K_{\text{off}}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance; has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one  
25 to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9; and has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12, such that said Behcet's disease is treated or prevented.

30 In another embodiment, the invention includes a method of treating or preventing Behcet's disease in a subject comprising administering a therapeutically effective amount a  $\text{TNF}\alpha$  antibody, or an antigen-binding fragment thereof, with a light chain

variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2, such that said Behcet's disease is treated or prevented. In one embodiment, the antibody, or antigen-binding fragment thereof, is D2E7.

5           In yet another embodiment, the invention includes a method for inhibiting human TNF $\alpha$  activity in a human subject suffering from vasculitis comprising administering a therapeutically effective amount of a TNF $\alpha$  antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF $\alpha$  with a  $K_D$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, both determined by  
10 surface plasmon resonance, and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-7}$  M or less.

          In one embodiment, the TNF $\alpha$  antibody, or antigen binding fragment thereof, is D2E7. In another embodiment, the vasculitis is a medium vessel disease. In still another embodiment, the medium vessel disease is Kawasaki's Disease. In one  
15 embodiment, the vasculitis is a small vessel disease. In another embodiment, the small vessel disease is Behcet's Syndrome. In still another embodiment, the vasculitis is selected from the group consisting of giant cell arteritis, temporal arteritis, polymyalgia rheumatica, Takayasu's disease, polyarteritis nodosa, Kawasaki's disease, Behcet's Syndrome, Wegener's granulomatosis, and Churg-Strauss syndrome.

20           The invention provides a method for inhibiting human TNF $\alpha$  activity in a human subject suffering from Behcet's disease, comprising administering a therapeutically effective amount of a TNF $\alpha$  antibody, or an antigen-binding fragment thereof, to the subject, wherein the antibody dissociates from human TNF $\alpha$  with a  $K_D$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, both determined by surface plasmon  
25 resonance, and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an  $IC_{50}$  of  $1 \times 10^{-7}$  M or less. In one embodiment, the antibody, or antigen binding fragment thereof, is D2E7.

          The invention also includes a method of treating a subject suffering from vasculitis comprising administering a therapeutically effective amount of D2E7, or an  
30 antigen-binding fragment thereof, to the subject, such that the vasculitis is treated.

In one embodiment, the vasculitis is a medium vessel disease. In still another embodiment, the medium vessel disease is Kawasaki's Disease. In one embodiment, the vasculitis is a small vessel disease. In another embodiment, the small vessel disease is Behcet's Syndrome. In still another embodiment, the vasculitis is selected from the group consisting of giant cell arteritis, temporal arteritis, polymyalgia rheumatica, Takayasu's disease, polyarteritis nodosa, Kawasaki's disease, Behcet's Syndrome, Wegener's granulomatosis, and Churg-Strauss syndrome.

In another embodiment, the invention includes a method of treating a subject suffering from Behcet's disease comprising administering a therapeutically effective amount of D2E7, or an antigen-binding fragment thereof, to the subject, such that said Behcet's disease is treated.

The invention also describes a kit comprising a pharmaceutical composition comprising a TNF $\alpha$  antibody, or an antigen binding portion thereof, and a pharmaceutically acceptable carrier; and instructions for administering to a subject the TNF $\alpha$  antibody pharmaceutical composition for treating a subject who is suffering from a vasculitis. In one embodiment, the TNF $\alpha$  antibody, or an antigen binding portion thereof, is D2E7.

#### **DETAILED DESCRIPTION OF THE INVENTION**

This invention pertains to methods of treating vasculitis in which TNF $\alpha$  activity, e.g., human TNF $\alpha$  activity, is detrimental. The methods include administering to the subject an effective amount of a TNF $\alpha$  antibody, such that the vasculitis is treated or prevented. In one embodiment, the TNF $\alpha$  antibody of the invention is administered to treat or prevent vasculitis. The invention also pertains to methods wherein the TNF $\alpha$  inhibitor is administered in combination with another therapeutic agent to treat a vasculitis. Various aspects of the invention relate to treatment with antibodies and antibody fragments, and pharmaceutical compositions comprising a TNF $\alpha$  inhibitor, and a pharmaceutically acceptable carrier for the treatment of a vasculitis.

*Definitions*

In order that the present invention may be more readily understood, certain terms are first defined.

The term "human TNF $\alpha$ " (abbreviated herein as hTNF $\alpha$ , or simply hTNF), as used herein, is intended to refer to a human cytokine that exists as a 17 kD secreted form and a 26 kD membrane associated form, the biologically active form of which is composed of a trimer of noncovalently bound 17 kD molecules. The structure of hTNF $\alpha$  is described further in, for example, Pennica, D., *et al.* (1984) *Nature* 312:724-729; Davis, J.M., *et al.* (1987) *Biochemistry* 26:1322-1326; and Jones, E.Y., *et al.* (1989) *Nature* 338:225-228. The term human TNF $\alpha$  is intended to include recombinant human TNF $\alpha$  (rhTNF $\alpha$ ), which can be prepared by standard recombinant expression methods or purchased commercially (R & D Systems, Catalog No. 210-TA, Minneapolis, MN). TNF $\alpha$  is also referred to as TNF.

The term "TNF $\alpha$  inhibitor" includes agents which inhibit TNF $\alpha$ . Examples of TNF $\alpha$  inhibitors include etanercept (Enbrel<sup>®</sup>, Amgen), infliximab (Remicade<sup>®</sup>, Johnson and Johnson), human anti-TNF monoclonal antibody (D2E7/HUMIRA<sup>®</sup>, Abbott Laboratories), CDP 571 (Celltech), and CDP 870 (Celltech) and other compounds which inhibit TNF $\alpha$  activity, such that when administered to a subject suffering from or at risk of suffering from a disorder in which TNF $\alpha$  activity is detrimental, the disorder is treated. In one embodiment, a TNF $\alpha$  inhibitor is a compound, excluding etanercept and infliximab, which inhibits TNF $\alpha$  activity. In another embodiment, the TNF $\alpha$  inhibitors of the invention are used to treat a TNF $\alpha$ -related disorder, as described in more detail in section II. In one embodiment, the TNF $\alpha$  inhibitor, excluding etanercept and infliximab, is used to treat a TNF $\alpha$ -related disorder. In another embodiment, the TNF $\alpha$  inhibitor, excluding etanercept and infliximab, is used to treat vasculitis. The term also includes each of the anti-TNF $\alpha$  human antibodies and antibody portions described herein as well as those described in U.S. Patent Nos. 6,090,382; 6,258,562; 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356.

The term "antibody", as used herein, is intended to refer to immunoglobulin molecules comprised of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant

region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into  
5 regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The antibodies of the invention are described in further detail in U.S.  
10 Patent Nos. 6,090,382; 6,258,562; and 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

The term "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the  
15 ability to specifically bind to an antigen (*e.g.*, hTNF $\alpha$ ). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')<sub>2</sub> fragment, a bivalent  
20 fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546 ), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of  
25 the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883). Such single chain  
30 antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. Other forms of single chain antibodies, such as diabodies are also encompassed. Diabodies are bivalent, bispecific antibodies in which VH and VL



domains are expressed on a single polypeptide chain, but using a linker that is too short to allow for pairing between the two domains on the same chain, thereby forcing the domains to pair with complementary domains of another chain and creating two antigen binding sites (see *e.g.*, Holliger, P., *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448; Poljak, R.J., *et al.* (1994) *Structure* 2:1121-1123). The antibody portions of the invention are described in further detail in U.S. Patent Nos. 6,090,382, 6,258,562, 6,509,015, and in U.S. Patent Application Serial Nos. 09/801185 and 10/302356, each of which is incorporated herein by reference in its entirety.

Binding fragments are produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins. Binding fragments include Fab, Fab', F(ab')<sub>2</sub>, Fabc, Fv, single chains, and single-chain antibodies. Other than "bispecific" or "bifunctional" immunoglobulins or antibodies, an immunoglobulin or antibody is understood to have each of its binding sites identical. A "bispecific" or "bifunctional antibody" is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, *Clin. Exp. Immunol.* 79:315-321 (1990); Kostelny *et al.*, *J. Immunol.* 148, 1547-1553 (1992).

A "conservative amino acid substitution", as used herein, is one in which one amino acid residue is replaced with another amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine).

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The human antibodies of the invention may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*), for

example in the CDRs and in particular CDR3. However, the term “human antibody”, as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

5           The term “recombinant human antibody”, as used herein, is intended to include all human antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell (described further below), antibodies isolated from a recombinant, combinatorial human antibody library (described further below), antibodies isolated  
10   from an animal (*e.g.*, a mouse) that is transgenic for human immunoglobulin genes (see *e.g.*, Taylor, L.D., *et al.* (1992) *Nucl. Acids Res.* 20:6287-6295) or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant human antibodies have variable and constant regions derived from human germline  
15   immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies are subjected to *in vitro* mutagenesis (or, when an animal transgenic for human Ig sequences is used, *in vivo* somatic mutagenesis) and thus the amino acid sequences of the VH and VL regions of the recombinant antibodies are sequences that, while derived from and related to human germline VH and VL sequences, may not  
20   naturally exist within the human antibody germline repertoire *in vivo*.

          An “isolated antibody”, as used herein, is intended to refer to an antibody that is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds hTNF $\alpha$  is substantially free of antibodies that specifically bind antigens other than hTNF $\alpha$ ). An isolated antibody that specifically  
25   binds hTNF $\alpha$  may, however, have cross-reactivity to other antigens, such as hTNF $\alpha$  molecules from other species (discussed in further detail below). Moreover, an isolated antibody may be substantially free of other cellular material and/or chemicals.

          A “neutralizing antibody”, as used herein (or an “antibody that neutralized hTNF $\alpha$  activity”), is intended to refer to an antibody whose binding to hTNF $\alpha$  results in  
30   inhibition of the biological activity of hTNF $\alpha$ . This inhibition of the biological activity of hTNF $\alpha$  can be assessed by measuring one or more indicators of hTNF $\alpha$  biological

activity, such as hTNF $\alpha$ -induced cytotoxicity (either *in vitro* or *in vivo*), hTNF $\alpha$ -induced cellular activation and hTNF $\alpha$  binding to hTNF $\alpha$  receptors. These indicators of hTNF $\alpha$  biological activity can be assessed by one or more of several standard *in vitro* or *in vivo* assays known in the art (see U.S. Patent No. 6,090,382). Preferably, the ability of an antibody to neutralize hTNF $\alpha$  activity is assessed by inhibition of hTNF $\alpha$ -induced cytotoxicity of L929 cells. As an additional or alternative parameter of hTNF $\alpha$  activity, the ability of an antibody to inhibit hTNF $\alpha$ -induced expression of ELAM-1 on HUVEC, as a measure of hTNF $\alpha$ -induced cellular activation, can be assessed.

The term “surface plasmon resonance”, as used herein, refers to an optical phenomenon that allows for the analysis of real-time biospecific interactions by detection of alterations in protein concentrations within a biosensor matrix, for example using the BIAcore system (Pharmacia Biosensor AB, Uppsala, Sweden and Piscataway, NJ). For further descriptions, see Example 1 and Jönsson, U., *et al.* (1993) *Ann. Biol. Clin.* 51:19-26; Jönsson, U., *et al.* (1991) *Biotechniques* 11:620-627; Johnsson, B., *et al.* (1995) *J. Mol. Recognit.* 8:125-131; and Johnsson, B., *et al.* (1991) *Anal. Biochem.* 198:268-277.

The term “K<sub>off</sub>”, as used herein, is intended to refer to the off rate constant for dissociation of an antibody from the antibody/antigen complex.

The term “K<sub>d</sub>”, as used herein, is intended to refer to the dissociation constant of a particular antibody-antigen interaction.

The term “IC<sub>50</sub>” as used herein, is intended to refer to the concentration of the inhibitor required to inhibit the biological endpoint of interest, e.g., neutralize cytotoxicity activity.

The term “nucleic acid molecule”, as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or double-stranded, but preferably is double-stranded DNA.

The term “isolated nucleic acid molecule”, as used herein in reference to nucleic acids encoding antibodies or antibody portions (*e.g.*, VH, VL, CDR3) that bind hTNF $\alpha$ , is intended to refer to a nucleic acid molecule in which the nucleotide sequences encoding the antibody or antibody portion are free of other nucleotide sequences encoding antibodies or antibody portions that bind antigens other than hTNF $\alpha$ , which

other sequences may naturally flank the nucleic acid in human genomic DNA. Thus, for example, an isolated nucleic acid of the invention encoding a VH region of an anti-hTNF $\alpha$  antibody contains no other sequences encoding other VH regions that bind antigens other than hTNF $\alpha$ .

5           The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors  
10 are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the  
15 expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However,  
20 the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

          The term "recombinant host cell" (or simply "host cell"), as used herein, is intended to refer to a cell into which a recombinant expression vector has been  
25 introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein.

30           The term "dosing", as used herein, refers to the administration of a substance (*e.g.*, an anti-TNF $\alpha$  antibody) to achieve a therapeutic objective (*e.g.*, the treatment of a TNF $\alpha$ -associated disorder).

The terms "biweekly dosing regimen", "biweekly dosing", and "biweekly administration", as used herein, refer to the time course of administering a substance (*e.g.*, an anti-TNF $\alpha$  antibody) to a subject to achieve a therapeutic objective (*e.g.*, the treatment of a TNF $\alpha$ -associated disorder). The biweekly dosing regimen is not intended  
5 to include a weekly dosing regimen. Preferably, the substance is administered every 9-19 days, more preferably, every 11-17 days, even more preferably, every 13-15 days, and most preferably, every 14 days.

The term "combination" as in the phrase "a first agent in combination with a second agent" includes co-administration of a first agent and a second agent, which for  
10 example may be dissolved or intermixed in the same pharmaceutically acceptable carrier, or administration of a first agent, followed by the second agent, or administration of the second agent, followed by the first agent. The present invention, therefore, includes methods of combination therapeutic treatment and combination pharmaceutical compositions. The term "combination therapy", as used herein, refers to the  
15 administration of two or more agents, *e.g.*, an anti-TNF $\alpha$  antibody and another drug, such as a DMARD or NSAID. The other drug(s) may be administered concomitant with, prior to, or following the administration of an anti-TNF $\alpha$  antibody.

The term "concomitant" as in the phrase "concomitant therapeutic treatment" includes administering an agent in the presence of a second agent. A concomitant  
20 therapeutic treatment method includes methods in which the first, second, third, or additional agents are co-administered. A concomitant therapeutic treatment method also includes methods in which the first or additional agents are administered in the presence of a second or additional agents, wherein the second or additional agents, for example, may have been previously administered. A concomitant therapeutic treatment method  
25 may be executed step-wise by different actors. For example, one actor may administer to a subject a first agent and a second actor may administer to the subject a second agent, and the administering steps may be executed at the same time, or nearly the same time, or at distant times, so long as the first agent (and additional agents) are after administration in the presence of the second agent (and additional agents). The actor  
30 and the subject may be the same entity (*e.g.*, human).

The term "kit" as used herein refers to a packaged product comprising components with which to administer the TNF $\alpha$  antibody of the invention for treatment

of a TNF $\alpha$ -related disorder. The kit preferably comprises a box or container that holds the components of the kit. The box or container is affixed with a label or a Food and Drug Administration approved protocol. The box or container holds components of the invention which are preferably contained within plastic, polyethylene, polypropylene,  
5 ethylene, or propylene vessels. The vessels can be capped-tubes or bottles. The kit can also include instructions for administering the TNF $\alpha$  antibody of the invention.

The term "vasculitis" or "vasculitides" as used interchangeably herein, refers to a group of disorders which are characterized by the inflammation of blood vessels. Blood vessels of all sizes may be affected, from the largest vessel in the body (the aorta) to the  
10 smallest blood vessels in the skin (capillaries). The size of blood vessel affected varies according to the specific type of vasculitis.

Various aspects of the invention are described in further detail herein.

#### I. TNF $\alpha$ Inhibitors of the Invention

15 This invention provides methods of treating vasculitides in which the administration of a TNF $\alpha$  inhibitor is beneficial, for example Behcet's disease. In one embodiment, these methods includes administration of isolated human antibodies, or antigen-binding portions thereof, that bind to human TNF $\alpha$  with high affinity, a low off rate and high neutralizing capacity. Preferably, the human antibodies of the invention  
20 are recombinant, neutralizing human anti-hTNF $\alpha$  antibodies. The most preferred recombinant, neutralizing antibody of the invention is referred to herein as D2E7 (the amino acid sequence of the D2E7 VL region is shown in SEQ ID NO: 1; the amino acid sequence of the D2E7 VH region is shown in SEQ ID NO: 2). D2E7 is also referred to as HUMIRA<sup>®</sup> and adalimumab. The properties of D2E7 have been described in Salfeld *et al.*, U.S. patent No. 6,090,382, which is incorporated by reference herein.  
25

In one embodiment, the treatment of the invention includes the administration of D2E7 antibodies and antibody portions, D2E7-related antibodies and antibody portions, and other human antibodies and antibody portions with equivalent properties to D2E7, such as high affinity binding to hTNF $\alpha$  with low dissociation kinetics and high  
30 neutralizing capacity. In one embodiment, the invention provides treatment with an isolated human antibody, or an antigen-binding portion thereof, that dissociates from

human TNF $\alpha$  with a  $K_d$  of  $1 \times 10^{-8}$  M or less and a  $K_{off}$  rate constant of  $1 \times 10^{-3}$  s $^{-1}$  or less, both determined by surface plasmon resonance, and neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an IC $_{50}$  of  $1 \times 10^{-7}$  M or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, dissociates  
5 from human TNF $\alpha$  with a  $K_{off}$  of  $5 \times 10^{-4}$  s $^{-1}$  or less, or even more preferably, with a  $K_{off}$  of  $1 \times 10^{-4}$  s $^{-1}$  or less. More preferably, the isolated human antibody, or antigen-binding portion thereof, neutralizes human TNF $\alpha$  cytotoxicity in a standard *in vitro* L929 assay with an IC $_{50}$  of  $1 \times 10^{-8}$  M or less, even more preferably with an IC $_{50}$  of  $1 \times 10^{-9}$  M or less and still more preferably with an IC $_{50}$  of  $1 \times 10^{-10}$  M or less. In a  
10 preferred embodiment, the antibody is an isolated human recombinant antibody, or an antigen-binding portion thereof.

It is well known in the art that antibody heavy and light chain CDR3 domains play an important role in the binding specificity/affinity of an antibody for an antigen. Accordingly, in another aspect, the invention pertains to methods of treating vasculitides  
15 in which the TNF $\alpha$  activity is detrimental by administering human antibodies that have slow dissociation kinetics for association with hTNF $\alpha$  and that have light and heavy chain CDR3 domains that structurally are identical to or related to those of D2E7. Position 9 of the D2E7 VL CDR3 can be occupied by Ala or Thr without substantially affecting the  $K_{off}$ . Accordingly, a consensus motif for the D2E7 VL CDR3 comprises  
20 the amino acid sequence: Q-R-Y-N-R-A-P-Y-(T/A) (SEQ ID NO: 3). Additionally, position 12 of the D2E7 VH CDR3 can be occupied by Tyr or Asn, without substantially affecting the  $K_{off}$ . Accordingly, a consensus motif for the D2E7 VH CDR3 comprises the amino acid sequence: V-S-Y-L-S-T-A-S-S-L-D-(Y/N) (SEQ ID NO: 4). Moreover, as demonstrated in Example 2, the CDR3 domain of the D2E7 heavy and light chains is  
25 amenable to substitution with a single alanine residue (at position 1, 4, 5, 7 or 8 within the VL CDR3 or at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 within the VH CDR3) without substantially affecting the  $K_{off}$ . Still further, the skilled artisan will appreciate that, given the amenability of the D2E7 VL and VH CDR3 domains to substitutions by alanine, substitution of other amino acids within the CDR3 domains may be possible  
30 while still retaining the low off rate constant of the antibody, in particular substitutions with conservative amino acids. Preferably, no more than one to five conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. More

preferably, no more than one to three conservative amino acid substitutions are made within the D2E7 VL and/or VH CDR3 domains. Additionally, conservative amino acid substitutions should not be made at amino acid positions critical for binding to hTNF $\alpha$ . Positions 2 and 5 of the D2E7 VL CDR3 and positions 1 and 7 of the D2E7 VH CDR3  
5 appear to be critical for interaction with hTNF $\alpha$  and thus, conservative amino acid substitutions preferably are not made at these positions (although an alanine substitution at position 5 of the D2E7 VL CDR3 is acceptable, as described above) (*see* U.S. Patent No. 6,090,382).

Accordingly, in another embodiment, the invention provides methods of treating  
10 vasculitides by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains the following characteristics:

- a) dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  rate constant of  $1 \times 10^{-3} \text{ s}^{-1}$  or less, as determined by surface plasmon resonance;
- 15 b) has a light chain CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8 or by one to five conservative amino acid substitutions at positions 1, 3, 4, 6, 7, 8 and/or 9;
- c) has a heavy chain CDR3 domain comprising the amino acid sequence of SEQ  
20 ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11 or by one to five conservative amino acid substitutions at positions 2, 3, 4, 5, 6, 8, 9, 10, 11 and/or 12.

More preferably, the antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  of  $5 \times 10^{-4} \text{ s}^{-1}$  or less. Even more preferably, the  
25 antibody, or antigen-binding portion thereof, dissociates from human TNF $\alpha$  with a  $K_{\text{off}}$  of  $1 \times 10^{-4} \text{ s}^{-1}$  or less.

In yet another embodiment, the invention provides methods of treating vasculitides by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a  
30 light chain variable region (LCVR) having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 3, or modified from SEQ ID NO: 3 by a single alanine substitution at position 1, 4, 5, 7 or 8, and with a heavy chain variable region (HCVR)



having a CDR3 domain comprising the amino acid sequence of SEQ ID NO: 4, or modified from SEQ ID NO: 4 by a single alanine substitution at position 2, 3, 4, 5, 6, 8, 9, 10 or 11. Preferably, the LCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 5 (*i.e.*, the D2E7 VL CDR2) and the HCVR further has a CDR2 domain comprising the amino acid sequence of SEQ ID NO: 6 (*i.e.*, the D2E7 VH CDR2). Even more preferably, the LCVR further has CDR1 domain comprising the amino acid sequence of SEQ ID NO: 7 (*i.e.*, the D2E7 VL CDR1) and the HCVR has a CDR1 domain comprising the amino acid sequence of SEQ ID NO: 8 (*i.e.*, the D2E7 VH CDR1). The framework regions for VL preferably are from the V<sub>K</sub>I human germline family, more preferably from the A20 human germline V<sub>K</sub> gene and most preferably from the D2E7 VL framework sequences shown in Figures 1A and 1B of U.S. Patent No. 6,090,382. The framework regions for VH preferably are from the V<sub>H</sub>3 human germline family, more preferably from the DP-31 human germline VH gene and most preferably from the D2E7 VH framework sequences shown in Figures 2A and 2B U.S. Patent No. 6,090,382.

Accordingly, in another embodiment, the invention provides methods of treating vasculitides by the administration of an isolated human antibody, or antigen-binding portion thereof. The antibody or antigen-binding portion thereof preferably contains a light chain variable region (LCVR) comprising the amino acid sequence of SEQ ID NO: 1 (*i.e.*, the D2E7 VL) and a heavy chain variable region (HCVR) comprising the amino acid sequence of SEQ ID NO: 2 (*i.e.*, the D2E7 VH). In certain embodiments, the antibody comprises a heavy chain constant region, such as an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region. Preferably, the heavy chain constant region is an IgG1 heavy chain constant region or an IgG4 heavy chain constant region. Furthermore, the antibody can comprise a light chain constant region, either a kappa light chain constant region or a lambda light chain constant region. Preferably, the antibody comprises a kappa light chain constant region. Alternatively, the antibody portion can be, for example, a Fab fragment or a single chain Fv fragment.

In still other embodiments, the invention provides methods of treating vasculitides in which the administration of an anti-TNF $\alpha$  antibody is beneficial administration of an isolated human antibody, or an antigen-binding portions thereof. The antibody or antigen-binding portion thereof preferably contains D2E7-related VL

and VH CDR3 domains, for example, antibodies, or antigen-binding portions thereof, with a light chain variable region (LCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, 5 SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 25 and SEQ ID NO: 26 or with a heavy chain variable region (HCVR) having a CDR3 domain comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 27, SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 30, SEQ ID NO: 31, SEQ ID NO: 10 32, SEQ ID NO: 33, SEQ ID NO: 34 and SEQ ID NO: 35.

In another embodiment, the TNF $\alpha$  inhibitor of the invention is etanercept (described in WO 91/03553 and WO 09/406476), infliximab (described in U.S. Patent No. 5,656,272), CDP571 (a humanized monoclonal anti-TNF- $\alpha$  IgG4 antibody), CDP 870 (a humanized monoclonal anti-TNF- $\alpha$  antibody fragment), 15 D2E7/HUMIRA<sup>®</sup> (a human anti-TNF mAb), soluble TNF receptor Type I, or a pegylated soluble TNF receptor Type I (PEGs TNF-R1).

The TNF $\alpha$  antibody of the invention can be modified. In some embodiments, the TNF $\alpha$  antibody or antigen binding fragments thereof, is chemically modified to provide a desired effect. For example, pegylation of antibodies and antibody fragments of the 20 invention may be carried out by any of the pegylation reactions known in the art, as described, for example, in the following references: *Focus on Growth Factors* 3:4-10 (1992); EP 0 154 316; and EP 0 401 384 (each of which is incorporated by reference herein in its entirety). Preferably, the pegylation is carried out via an acylation reaction or an alkylation reaction with a reactive polyethylene glycol molecule (or an analogous 25 reactive water-soluble polymer). A preferred water-soluble polymer for pegylation of the antibodies and antibody fragments of the invention is polyethylene glycol (PEG). As used herein, "polyethylene glycol" is meant to encompass any of the forms of PEG that have been used to derivatize other proteins, such as mono (Cl-ClO) alkoxy- or aryloxy-polyethylene glycol.

30 Methods for preparing pegylated antibodies and antibody fragments of the invention will generally comprise the steps of (a) reacting the antibody or antibody fragment with polyethylene glycol, such as a reactive ester or aldehyde derivative of

PEG, under conditions whereby the antibody or antibody fragment becomes attached to one or more PEG groups, and (b) obtaining the reaction products. It will be apparent to one of ordinary skill in the art to select the optimal reaction conditions or the acylation reactions based on known parameters and the desired result.

5           Pegylated antibodies and antibody fragments may generally be used to treat vasculitis by administration of the TNF $\alpha$  antibodies and antibody fragments described herein. Generally the pegylated antibodies and antibody fragments have increased half-life, as compared to the nonpegylated antibodies and antibody fragments. The pegylated antibodies and antibody fragments may be employed alone, together, or in combination  
10 with other pharmaceutical compositions.

          In yet another embodiment of the invention, TNF $\alpha$  antibodies or fragments thereof can be altered wherein the constant region of the antibody is modified to reduce at least one constant region-mediated biological effector function relative to an unmodified antibody. To modify an antibody of the invention such that it exhibits  
15 reduced binding to the Fc receptor, the immunoglobulin constant region segment of the antibody can be mutated at particular regions necessary for Fc receptor (FcR) interactions (see *e.g.*, Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. *et al.* (1991) *J. of Immunol.* 147:2657-2662). Reduction in FcR binding ability of the antibody may also reduce other effector functions which rely on  
20 FcR interactions, such as opsonization and phagocytosis and antigen-dependent cellular cytotoxicity.

          An antibody or antibody portion of the invention can be derivatized or linked to another functional molecule (*e.g.*, another peptide or protein). Accordingly, the antibodies and antibody portions of the invention are intended to include derivatized and  
25 otherwise modified forms of the human anti-hTNF $\alpha$  antibodies described herein, including immunoadhesion molecules. For example, an antibody or antibody portion of the invention can be functionally linked (by chemical coupling, genetic fusion, noncovalent association or otherwise) to one or more other molecular entities, such as another antibody (*e.g.*, a bispecific antibody or a diabody), a detectable agent, a cytotoxic  
30 agent, a pharmaceutical agent, and/or a protein or peptide that can mediate association of the antibody or antibody portion with another molecule (such as a streptavidin core region or a polyhistidine tag).

One type of derivatized antibody is produced by crosslinking two or more antibodies (of the same type or of different types, *e.g.*, to create bispecific antibodies). Suitable crosslinkers include those that are heterobifunctional, having two distinctly reactive groups separated by an appropriate spacer (*e.g.*, m-maleimidobenzoyl-N-  
5 hydroxysuccinimide ester) or homobifunctional (*e.g.*, disuccinimidyl suberate). Such linkers are available from Pierce Chemical Company, Rockford, IL.

Useful detectable agents with which an antibody or antibody portion of the invention may be derivatized include fluorescent compounds. Exemplary fluorescent detectable agents include fluorescein, fluorescein isothiocyanate, rhodamine, 5-  
10 dimethylamine-1-naphthalenesulfonyl chloride, phycoerythrin and the like. An antibody may also be derivatized with detectable enzymes, such as alkaline phosphatase, horseradish peroxidase, glucose oxidase and the like. When an antibody is derivatized with a detectable enzyme, it is detected by adding additional reagents that the enzyme uses to produce a detectable reaction product. For example, when the detectable agent  
15 horseradish peroxidase is present, the addition of hydrogen peroxide and diaminobenzidine leads to a colored reaction product, which is detectable. An antibody may also be derivatized with biotin, and detected through indirect measurement of avidin or streptavidin binding.

An antibody, or antibody portion, of the invention can be prepared by  
20 recombinant expression of immunoglobulin light and heavy chain genes in a host cell. To express an antibody recombinantly, a host cell is transfected with one or more recombinant expression vectors carrying DNA fragments encoding the immunoglobulin light and heavy chains of the antibody such that the light and heavy chains are expressed in the host cell and, preferably, secreted into the medium in which the host cells are  
25 cultured, from which medium the antibodies can be recovered. Standard recombinant DNA methodologies are used to obtain antibody heavy and light chain genes, incorporate these genes into recombinant expression vectors and introduce the vectors into host cells, such as those described in Sambrook, Fritsch and Maniatis (eds), *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor, N.Y.,  
30 (1989), Ausubel, F.M. *et al.* (eds.) *Current Protocols in Molecular Biology*, Greene Publishing Associates, (1989) and in U.S. Patent No. 4,816,397 by Boss *et al.*

To express D2E7 or a D2E7-related antibody, DNA fragments encoding the light and heavy chain variable regions are first obtained. These DNAs can be obtained by amplification and modification of germline light and heavy chain variable sequences using the polymerase chain reaction (PCR). Germline DNA sequences for human heavy and light chain variable region genes are known in the art (see *e.g.*, the "Vbase" human germline sequence database; see also Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242; Tomlinson, I.M., *et al.* (1992) "The Repertoire of Human Germline V<sub>H</sub> Sequences Reveals about Fifty Groups of V<sub>H</sub> Segments with Different Hypervariable Loops" *J. Mol. Biol.* 227:776-798; and Cox, J.P.L. *et al.* (1994) "A Directory of Human Germ-line V<sub>78</sub> Segments Reveals a Strong Bias in their Usage" *Eur. J. Immunol.* 24:827-836; the contents of each of which are expressly incorporated herein by reference). To obtain a DNA fragment encoding the heavy chain variable region of D2E7, or a D2E7-related antibody, a member of the V<sub>H</sub>3 family of human germline VH genes is amplified by standard PCR. Most preferably, the DP-31 VH germline sequence is amplified. To obtain a DNA fragment encoding the light chain variable region of D2E7, or a D2E7-related antibody, a member of the V<sub>K</sub>I family of human germline VL genes is amplified by standard PCR. Most preferably, the A20 VL germline sequence is amplified. PCR primers suitable for use in amplifying the DP-31 germline VH and A20 germline VL sequences can be designed based on the nucleotide sequences disclosed in the references cited *supra*, using standard methods.

Once the germline VH and VL fragments are obtained, these sequences can be mutated to encode the D2E7 or D2E7-related amino acid sequences disclosed herein. The amino acid sequences encoded by the germline VH and VL DNA sequences are first compared to the D2E7 or D2E7-related VH and VL amino acid sequences to identify amino acid residues in the D2E7 or D2E7-related sequence that differ from germline. Then, the appropriate nucleotides of the germline DNA sequences are mutated such that the mutated germline sequence encodes the D2E7 or D2E7-related amino acid sequence, using the genetic code to determine which nucleotide changes should be made. Mutagenesis of the germline sequences is carried out by standard methods, such as PCR-mediated mutagenesis (in which the mutated nucleotides are incorporated into the PCR primers such that the PCR product contains the mutations) or site-directed mutagenesis.

Once DNA fragments encoding D2E7 or D2E7-related VH and VL segments are obtained (by amplification and mutagenesis of germline VH and VL genes, as described above), these DNA fragments can be further manipulated by standard recombinant DNA techniques, for example to convert the variable region genes to full-length antibody chain genes, to Fab fragment genes or to a scFv gene. In these manipulations, a VL- or VH-encoding DNA fragment is operatively linked to another DNA fragment encoding another protein, such as an antibody constant region or a flexible linker. The term "operatively linked", as used in this context, is intended to mean that the two DNA fragments are joined such that the amino acid sequences encoded by the two DNA fragments remain in-frame.

The isolated DNA encoding the VH region can be converted to a full-length heavy chain gene by operatively linking the VH-encoding DNA to another DNA molecule encoding heavy chain constant regions (CH1, CH2 and CH3). The sequences of human heavy chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The heavy chain constant region can be an IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM or IgD constant region, but most preferably is an IgG1 or IgG4 constant region. For a Fab fragment heavy chain gene, the VH-encoding DNA can be operatively linked to another DNA molecule encoding only the heavy chain CH1 constant region.

The isolated DNA encoding the VL region can be converted to a full-length light chain gene (as well as a Fab light chain gene) by operatively linking the VL-encoding DNA to another DNA molecule encoding the light chain constant region, CL. The sequences of human light chain constant region genes are known in the art (see *e.g.*, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242) and DNA fragments encompassing these regions can be obtained by standard PCR amplification. The light chain constant region can be a kappa or lambda constant region, but most preferably is a kappa constant region.

To create a scFv gene, the VH- and VL-encoding DNA fragments are operatively linked to another fragment encoding a flexible linker, *e.g.*, encoding the amino acid

sequence (Gly<sub>4</sub>-Ser)<sub>3</sub>, such that the VH and VL sequences can be expressed as a contiguous single-chain protein, with the VL and VH regions joined by the flexible linker (see *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883; McCafferty *et al.*, *Nature* (1990) 348:552-554).

5           To express the antibodies, or antibody portions of the invention, DNAs encoding partial or full-length light and heavy chains, obtained as described above, are inserted into expression vectors such that the genes are operatively linked to transcriptional and translational control sequences. In this context, the term "operatively linked" is intended to mean that an antibody gene is ligated into a vector such that transcriptional and  
10 translational control sequences within the vector serve their intended function of regulating the transcription and translation of the antibody gene. The expression vector and expression control sequences are chosen to be compatible with the expression host cell used. The antibody light chain gene and the antibody heavy chain gene can be inserted into separate vector or, more typically, both genes are inserted into the same  
15 expression vector. The antibody genes are inserted into the expression vector by standard methods (*e.g.*, ligation of complementary restriction sites on the antibody gene fragment and vector, or blunt end ligation if no restriction sites are present). Prior to insertion of the D2E7 or D2E7-related light or heavy chain sequences, the expression vector may already carry antibody constant region sequences. For example, one  
20 approach to converting the D2E7 or D2E7-related VH and VL sequences to full-length antibody genes is to insert them into expression vectors already encoding heavy chain constant and light chain constant regions, respectively, such that the VH segment is operatively linked to the CH segment(s) within the vector and the VL segment is operatively linked to the CL segment within the vector. Additionally or alternatively,  
25 the recombinant expression vector can encode a signal peptide that facilitates secretion of the antibody chain from a host cell. The antibody chain gene can be cloned into the vector such that the signal peptide is linked in-frame to the amino terminus of the antibody chain gene. The signal peptide can be an immunoglobulin signal peptide or a heterologous signal peptide (*i.e.*, a signal peptide from a non-immunoglobulin protein).  
30           In addition to the antibody chain genes, the recombinant expression vectors of the invention carry regulatory sequences that control the expression of the antibody chain genes in a host cell. The term "regulatory sequence" is intended to include

promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals) that control the transcription or translation of the antibody chain genes. Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). It  
5 will be appreciated by those skilled in the art that the design of the expression vector, including the selection of regulatory sequences may depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, *etc.* Preferred regulatory sequences for mammalian host cell expression include viral elements that direct high levels of protein expression in mammalian cells, such as  
10 promoters and/or enhancers derived from cytomegalovirus (CMV) (such as the CMV promoter/enhancer), Simian Virus 40 (SV40) (such as the SV40 promoter/enhancer), adenovirus, (*e.g.*, the adenovirus major late promoter (AdMLP)) and polyoma. For further description of viral regulatory elements, and sequences thereof, see *e.g.*, U.S. Patent No. 5,168,062 by Stinski, U.S. Patent No. 4,510,245 by Bell *et al.* and U.S. Patent  
15 No. 4,968,615 by Schaffner *et al.*

In addition to the antibody chain genes and regulatory sequences, the recombinant expression vectors of the invention may carry additional sequences, such as sequences that regulate replication of the vector in host cells (*e.g.*, origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host  
20 cells into which the vector has been introduced (see *e.g.*, U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017, all by Axel *et al.*). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase (DHFR) gene (for use in *dhfr*<sup>-</sup> host cells with  
25 methotrexate selection/amplification) and the *neo* gene (for G418 selection).

For expression of the light and heavy chains, the expression vector(s) encoding the heavy and light chains is transfected into a host cell by standard techniques. The various forms of the term “transfection” are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or  
30 eukaryotic host cell, *e.g.*, electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is theoretically possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of



antibodies in eukaryotic cells, and most preferably mammalian host cells, is the most preferred because such eukaryotic cells, and in particular mammalian cells, are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody. Prokaryotic expression of antibody genes has been reported to be ineffective for production of high yields of active antibody (Boss, M.A. and Wood, C. R. (1985) *Immunology Today* 6:12-13).

Preferred mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin, (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, *e.g.*, as described in R.J. Kaufman and P.A. Sharp (1982) *Mol. Biol.* 159:601-621), NS0 myeloma cells, COS cells and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

Host cells can also be used to produce portions of intact antibodies, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure are within the scope of the present invention. For example, it may be desirable to transfect a host cell with DNA encoding either the light chain or the heavy chain (but not both) of an antibody of this invention. Recombinant DNA technology may also be used to remove some or all of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to hTNF $\alpha$ . The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention and the other heavy and light chain are specific for an antigen other than hTNF $\alpha$  by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells

by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows  
5 for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are culture to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture  
10 the host cells and recover the antibody from the culture medium.

Recombinant human antibodies of the invention in addition to D2E7 or an antigen binding portion thereof, or D2E7-related antibodies disclosed herein can be isolated by screening of a recombinant combinatorial antibody library, preferably a scFv phage display library, prepared using human VL and VH cDNAs prepared from mRNA  
15 derived from human lymphocytes. Methodologies for preparing and screening such libraries are known in the art. In addition to commercially available kits for generating phage display libraries (*e.g.*, the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*<sup>TM</sup> phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating  
20 and screening antibody display libraries can be found in, for example, Ladner *et al.* U.S. Patent No. 5,223,409; Kang *et al.* PCT Publication No. WO 92/18619; Dower *et al.* PCT Publication No. WO 91/17271; Winter *et al.* PCT Publication No. WO 92/20791; Markland *et al.* PCT Publication No. WO 92/15679; Breitling *et al.* PCT Publication No. WO 93/01288; McCafferty *et al.* PCT Publication No. WO 92/01047; Garrard *et al.*  
25 PCT Publication No. WO 92/09690; Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; McCafferty *et al.*, *Nature* (1990) 348:552-554; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.*  
30 (1991) *Bio/Technology* 9:1373-1377; Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982. Methods of isolating human antibodies with high affinity and a low off rate constant for hTNF $\alpha$  are described in U.S.

Patent Nos. 6,090,382, 6,258,562, and 6,509,015, each of which is incorporated by reference herein.

## II. Uses of TNF $\alpha$ Inhibitors of the Invention

5           The invention features a method for treating or preventing vasculitis in which TNF $\alpha$  activity is detrimental, comprising administering to a subject an effective amount of a TNF $\alpha$  inhibitor, such that said disorder is treated or prevented. In one embodiment, the vasculitis is giant cell arteritis, temporal arteritis, polymyalgia rheumatica, Takayasu's disease, polyarteritis nodosa, Kawasaki's disease, Behcet's Syndrome,  
10 Wegener's granulomatosis, and Churg-Strauss syndrome. In one embodiment, the TNF $\alpha$  inhibitor is D2E7, also referred to as HUMIRA<sup>®</sup> (adalimumab).

          In an embodiment, the invention provides a method for inhibiting TNF $\alpha$  activity in a subject suffering from a vasculitis in which TNF $\alpha$  activity is detrimental. TNF $\alpha$  has been implicated in the pathophysiology of a variety of vasculitides, (see *e.g.*, Deguchi *et al.* (1989) Lancet. 2:745). The invention provides methods for TNF $\alpha$  activity in a  
15 subject suffering from vasculitis, which method comprises administering to the subject an antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention such that TNF $\alpha$  activity in the subject with or at risk of having vasculitis, is inhibited. The invention also provides methods for inhibiting or decreasing TNF $\alpha$  activity in a subject with  
20 vasculitis, comprising administering to the subject an antibody, or antibody portion, or other TNF $\alpha$  inhibitor of the invention such that TNF $\alpha$  activity in the subject is inhibited or decreased. Preferably, the TNF $\alpha$  is human TNF $\alpha$  and the subject is a human subject. Alternatively, the subject can be a mammal expressing a TNF $\alpha$  with which an antibody of the invention cross-reacts. Still further the subject can be a mammal into which has  
25 been introduced hTNF $\alpha$  (*e.g.*, by administration of hTNF $\alpha$  or by expression of an hTNF $\alpha$  transgene). Alternatively, the subject can be a mammal expressing a TNF $\alpha$  with which an antibody of the invention cross-reacts. An antibody of the invention can be administered to a human subject for therapeutic purposes (discussed further below). Moreover, an antibody of the invention can be administered to a non-human mammal  
30 expressing a TNF $\alpha$  with which the antibody cross-reacts (*e.g.*, a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter,

such animal models may be useful for evaluating the therapeutic efficacy of antibodies of the invention (*e.g.*, testing of dosages and time courses of administration). Examples of animal models which are used to study the efficacy of a therapeutic agent in the treatment of vasculitis includes the mouse HSV model (Behcet's disease), the mouse *L. casei* model (Kawasaki's disease), and the mouse ANCA model (Kawasaki's disease).  
5 Other models of vasculitis include the McH5-*lpr/lpr* strain (Nose, M., *et al.* (1996) *Am. J. Path.* 149:1763) and the SCG/Kj strain of mice (Kinjoh, *et al.* (1993) *Proc. Natl. Acad. Sci., USA* 90:3413). These mice strains spontaneously develop crescentic glomerulonephritis and necrotizing vasculitis of the small arteries and arterioles of the  
10 spleen, stomach, heart, uterus and ovaries. These animals develop hypergammaglobulinemia and ANCA autoantibodies that react with myeloperoxidase (MPO). Additionally, immunization of rats with human MPO results in ANCA-associated necrotizing crescentic glomerulonephritis (Brouwer, E., *et al.* (1993) *J. Exp. Med.* 177:905). Each of these animal models can be used to test the efficacy of the  
15 TNF $\alpha$  antibody of the invention.

As used herein, the term "a vasculitis in which TNF $\alpha$  activity is detrimental" is intended to include vasculitis in which the presence of TNF $\alpha$  in a subject suffering from the disorder has been shown to be or is suspected of being either responsible for the pathophysiology of the disorder or a factor that contributes to a worsening of the  
20 disorder. Such disorders may be evidenced, for example, by an increase in the concentration of TNF $\alpha$  in a biological fluid of a subject suffering from the disorder (*e.g.*, an increase in the concentration of TNF $\alpha$  in serum, plasma, synovial fluid, *etc.* of the subject), which can be detected, for example, using an anti-TNF $\alpha$  antibody as described above.

25 There are numerous examples of vasculitides in which TNF $\alpha$  activity is detrimental, including Behcet's disease. The use of the antibodies, antibody portions, and other TNF $\alpha$  inhibitors of the invention in the treatment of specific vasculitides are discussed further below. In certain embodiments, the antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention is administered to the subject in combination with  
30 another therapeutic agent, as described below

The antibody of the invention can be used to treat vasculitis in which TNF $\alpha$  activity is detrimental, wherein inhibition of TNF $\alpha$  activity is expected to alleviate the symptoms and/or progression of the vasculitis or to prevent the vasculitis. Subjects suffering from or at risk of developing vasculitis can be identified through clinical  
5 symptoms and tests. For example, subjects with vasculitides often develop antibodies to certain proteins in the cytoplasm of neutrophils, antineutrophil cytoplasmic antibodies (ANCA). Thus, in some instances, vasculitides may be evidenced by tests (e.g., ELISA), which measure ANCA presence.

Vasculitis and its consequences may be the sole manifestation of disease or it  
10 may be a secondary component of another primary disease. Vasculitis may be confined to a single organ or it may simultaneously affect several organs. and depending on the syndrome, arteries and veins of all sizes can be affected. Vasculitis can affect any organ in the body.

In vasculitis, the vessel lumen is usually compromised, which is associated with  
15 ischemia of the tissues supplied by the involved vessel. The broad range of disorders that may result from this process is due to the fact that any type, size and location of vessel (e.g., artery, vein, arteriole, venule, capillary) can be involved. Vasculitides are generally classified according to the size of the affected vessels, as described below. It should be noted that some small and large vessel vasculitides may involve medium-sized  
20 arteries; but large and medium-sized vessel vasculitides do not involve vessels smaller than arteries. Large vessel disease includes, but is not limited to, giant cell arteritis, also known as temporal arteritis or cranial arteritis, polymyalgia rheumatica, and Takayasu's disease or arteritis, which is also known as aortic arch syndrome, young female arteritis and Pulseless disease. Medium vessel disease includes, but is not limited to, classic  
25 polyarteritis nodosa and Kawasaki's disease, also known as mucocutaneous lymph node syndrome. Non-limiting examples of small vessel disease are Behcet's Syndrome, Wegner's granulomatosis, microscopic polyangitis, hypersensitivity vasculitis, also known as cutaneous vasculitis, small vessel vasculitis, Henoch-Schonlein purpura, allergic granulomatosis and vasculitis, also known as Churg Strauss syndrome. Other  
30 vasculitides include, but are not limited to, isolated central nervous system vasculitis, and thromboangitis obliterans, also known as Buerger's disease. Classic Polyarteritis nodosa (PAN), microscopic PAN, and allergic granulomatosis are also often grouped

together and are called the systemic necrotizing vasculitides. A further description of vasculitis is described below:

A. *Large vessel vasculitis*

5 In one embodiment, the TNF $\alpha$  antibody of the invention is used to treat subjects who have large vessel vasculitis. The term "large vessel(s)" as used herein, refers to the aorta and the largest branches directed toward major body regions. Large vessels include, for example, the aorta, and its branches and corresponding veins, *e.g.*, the subclavian artery; the brachiocephalic artery; the common carotid artery; the innominate  
10 vein; internal and external jugular veins; the pulmonary arteries and veins; the venae cavae; the renal arteries and veins; the femoral arteries and veins; and the carotid arteries. Examples of large vessel vasculitides are described below.

i. *Giant cell arteritis (GCA)*

15 Tumor necrosis factor has been implicated in the pathophysiology of giant cell arteritis (Sneller, M.C. (2002) *Cleve. Clin. J. Med.* 69:SII40-3; Schett, G., *et al.* (2002) *Ann. Rheum. Dis.* 61:463). Giant cell arteritis (GCA), refers to a vasculitis involving inflammation and damage to blood vessels, particularly the large or medium arteries that branch from the external carotid artery of the neck. GCA is also referred to as temporal  
20 arteritis or cranial arteritis, and is the most common primary vasculitis in the elderly. It almost exclusively affects individuals over 50 years of age, however, there are well-documented cases of patients 40 years and younger. GCA usually affects extracranial arteries. GCA can affect the branches of the carotid arteries, including the temporal artery. GCA is also a systemic disease which can involve arteries in multiple locations.  
25 Histopathologically, GCA is a panarteritis with inflammatory mononuclear cell infiltrates within the vessel wall with frequent Langhans type giant cell formation. There is proliferation of the intima, granulomatous inflammation and fragmentation of the internal elastic lamina. The pathological findings in organs is the result of ischemia related to the involved vessels.

30 Patients suffering from GCA exhibit certain clinical symptoms, including fever, headache, anemia and high erythrocyte sedimentation rate (ESR). Other typical indications of GCA include jaw or tongue claudication, scalp tenderness, constitutional

symptoms, pale optic disc edema (particularly 'chalky white' disc edema), and vision disturbances. The diagnosis is confirmed by temporal artery biopsy.

ii. *Polymyalgia rheumatica*

5 Tumor necrosis factor has been implicated in the pathophysiology of polymyalgia rheumatica (Straub, R.H., *et al.* (2002) *Rheumatology* (Oxford) 41:423; Uddhammar, A., *et al.* (1998) *Br. J. Rheumatol.*37:766). Polymyalgia rheumatica refers to a rheumatic disorder that is associated with moderate to severe muscle pain and stiffness in the neck, shoulder, and hip, most noticeable in the morning. IL-6 and IL-  
10 1 $\beta$  expression has also been detected in a majority of the circulating monocytes in patients with the polymyalgia rheumatica. Polymyalgia rheumatica may occur independently, or it may coexist with or precede GCA, which is an inflammation of blood vessels.

15 iii. *Takayasu's arteritis*

Tumor necrosis factor has been implicated in the pathophysiology of Takayasu's arteritis (Kobayashi, Y. and Numano, F. (2002) *Intern. Med.* 41:44; Fraga, A. and Medina F. (2002) *Curr. Rheumatol. Rep.*4:30). Takayasu's arteritis refers to a  
vasculitis characterized by an inflammation of the aorta and its major branches.  
20 Takayasu's arteritis (also known as Aortic arch syndrome, young female arteritis and Pulseless disease) affects the thoracic and abdominal aorta and its main branches or the pulmonary arteries. Fibrotic thickening of the aortic wall and its branches (*e.g.*, carotid, innominate, and subclavian arteries) can lead to reduction of lumen size of vessels that arise from the aortic arch. This condition also typically affects the renal arteries.

25 Takayasu's arteritis primarily affects young women, usually aged 20-40 years old, particularly of Asian descent, and may be manifested by malaise, arthralgias and the gradual onset of extremity claudication. Most patients have asymmetrically reduced pulses, usually along with a blood pressure differential in the arms. Coronary and/or renal artery stenosis may occur.

30 The clinical features of Takayasu's arteritis may be divided into the features of the early inflammatory disease and the features of the later disease. The clinical features of the early inflammatory stage of Takayasu's disease are: malaise, low grade fever,

weight loss, myalgia, arthralgia, and erythema multiforme. Later stages of Takayasu's disease are characterised by fibrotic stenosis of arteries and thrombosis. The main resulting clinical features are ischaemic phenomena, *e.g.* weak and asymmetrical arterial pulses, blood pressure discrepancy between the arms, visual disturbance, *e.g.* scotomata and hemianopia, other neurological features including vertigo and syncope, hemiparesis or stroke. The clinical features result from ischaemia due to arterial stenosis and thrombosis.

B. *Medium vessel disease*

In one embodiment, the TNF $\alpha$  antibody of the invention is used to treat subjects who have medium vessel vasculitis. The term "medium vessel(s)" is used to refer to those blood vessels which are the main visceral arteries. Examples of medium vessels include the mesenteric arteries and veins, the iliac arteries and veins, and the maxillary arteries and veins. Examples of medium vessel vasculitides are described below.

i. *Polyarteritis nodosa*

Tumor necrosis factor has been implicated in the pathophysiology of polyarteritis nodosa (DiGirolamo, N., *et al.* (1997) *J. Leukoc. Biol.* 61:667). Polyarteritis nodosa, or periarteritis nodosa refers to vasculitis which is a serious blood vessel disease in which small and medium-sized arteries become swollen and damaged because they are attacked by rogue immune cells. Polyarteritis nodosa usually affects adults more frequently than children. It damages the tissues supplied by the affected arteries because they don't receive enough oxygen and nourishment without a proper blood supply.

Symptoms which are exhibited in patients with polyarteritis nodosa generally result from damage to affected organs, often the skin, heart, kidneys, and nervous system. Generalized symptoms of polyarteritis nodosa include fever, fatigue, weakness, loss of appetite, and weight loss. Muscle aches (myalgia) and joint aches (arthralgia) are common. The skin of subjects with polyarteritis nodosa may also show rashes, swelling, ulcers, and lumps (nodular lesions).

Classic PAN (polyarteritis nodosa) is a systemic arteritis of small to medium muscular arteritis in which involvement of renal and visceral arteries is common. Abdominal vessels have aneurysms or occlusions in 50% of PAN patients. Classic PAN



does not involve the pulmonary arteries although the bronchial vessels may be involved. Granulomas, significant eosinophilia and an allergic diathesis are not part of the syndrome. Although any organ system may be involved, the most common manifestations include peripheral neuropathy, mononeuritis multiplex, intestinal  
5 ischemia, renal ischemia, testicular pain and livedo reticularis.

ii. *Kawasaki's disease*

Tumor necrosis factor has been implicated in the pathophysiology of Kawasaki's disease (Sundel, R.P. (2002) *Curr. Rheumatol. Rep.* 4:474; Gedalia, A.(2002) *Curr.*  
10 *Rheumatol. Rep.* 4:25). Although the cause of Kawasaki's disease is unknown, it is associated with acute inflammation of the coronary arteries, suggesting that the tissue damage associated with this disease may be mediated by proinflammatory agents such as  $\text{TNF}\alpha$ . Kawasaki's disease refers to a vasculitis that affects the mucus membranes, lymph nodes, lining of the blood vessels, and the heart. Kawasaki's disease is also often  
15 referred to as mucocutaneous lymph node syndrome, mucocutaneous lymph node disease, and infantile polyarteritis. Subjects afflicted with Kawasaki's disease develop vasculitis often involving the coronary arteries which can lead to myocarditis and pericarditis. Often as the acute inflammation diminishes, the coronary arteries may develop aneurysm, thrombosis, and lead to myocardial infarction.

20 Kawasaki's disease is a febrile systemic vasculitis associated with edema in the palms and the soles of the feet, with enlargement of cervical lymph nodes, cracked lips and "strawberry tongue". Although the inflammatory response is found in vessels throughout the body, the most common site of end-organ damage is the coronary arteries. Kawasaki's Disease predominantly affects children under the age of 5. The  
25 highest incidence is in Japan but is becoming increasingly recognized in the West and is now the leading cause of acquired heart disease in US children. The most serious complication of Kawasaki disease is coronary arteritis and aneurysm formation that occurs in a third of untreated patients.

30 A. *Small vessel disease*

In one embodiment, the  $\text{TNF}\alpha$  antibody of the invention is used to treat subjects who have small vessel vasculitis. The term "small vessel(s)" is used to refer to

arterioles, venules and capillaries. Arterioles are arteries that contain only 1 or 2 layers of smooth muscle cells and are terminal to and continuous with the capillary network. Venules carry blood from the capillary network to veins and capillaries connect arterioles and venules. Examples of small vessel vasculitides are described below.

5

i. *Behcet's disease*

Tumor necrosis factor has been implicated in the pathophysiology of Behcet's disease (Sfikakis, P.P. (2002) *Ann. Rheum. Dis.* 61:ii51-3; Dogan, D. and Farah, C. (2002) *Ophthalmologia.* 52:23). Behcet's disease is a chronic disorder that involves  
10 inflammation of blood vessels throughout the body. Behcet's disease may also cause various types of skin lesions, arthritis, bowel inflammation, and meningitis (inflammation of the membranes of the brain and spinal cord). As a result of Behcet's disease, the subject with the disorder may have inflammation in tissues and organs throughout the body, including the gastrointestinal tract, central nervous system,  
15 vascular system, lungs, and kidneys. Behcet's disease is three times more common in males than females and is more common in the east Mediterranean and Japan.

Subjects who have Behcet's disease may show clinical symptoms including recurrent oral ulcers (resembling canker sores), recurrent genital ulcers, and eye inflammation. Serum levels of TNF $\alpha$ , IL-8, IL-1, IL-6 INF- $\gamma$  and IL-12 are elevated in  
20 Behcet's patients, and the production of these factors has been shown to be elevated in the monocytes of Behcet's patients (see, e.g., *Inflammatory Disease of Blood Vessels* (2001) Marcel Dekker, Inc., eds. G.S. Hoffman and C.M. Weyand, p. 473).

ii. *Wegener's granulomatosis*

25 Tumor necrosis factor has been implicated in the pathophysiology of Wegener's granulomatosis (Marquez, J., et al. (2003) *Curr. Rheumatol. Rep.* 5:128; Harman, L.E. and Margo, C.E. (1998) *Surv. Ophthalmol.* 42:458). Wegener's granulomatosis refers to a vasculitis that causes inflammation of blood vessels in the upper respiratory tract (nose, sinuses, ears), lungs, and kidneys. Wegener's granulomatosis is also referred to as  
30 midline granulomatosis. Wegener's granulomatosis includes a granulomatous inflammation involving the respiratory tract, and necrotizing vasculitis affecting small to medium-sized vessels. Subjects who have Wegener's granulomatosis often also have

arthritis (joint inflammation). Glomerulonephritis may also be present in affected subjects, but virtually any organ may be involved.

Patients affected with Wegener's granulomatosis typically show clinical symptoms comprising recurrent sinusitis or epistaxis, mucosal ulcerations, otitis media, cough, hemoptysis and dyspnea. The first symptoms of Wegener's granulomatosis frequently include upper respiratory tract symptoms, joint pains, weakness, and tiredness.

iii. *Churg-Strauss syndrome*

Tumor necrosis factor has been implicated in the pathophysiology of Churg-Strauss syndrome (Gross, W.L (2002) *Curr. Opin. Rheumatol.* 14:11; Churg, W.A.(2001) *Mod. Pathol.* 14:1284). Churg-Strauss syndrome refers to a vasculitis that is systemic and shows early manifestation signs of asthma and eosinophilia. Churg-Strauss syndrome is also referred to as allergic granulomatosis and angiitis, and occurs in the setting of allergic rhinitis, asthma and eosinophilia. Sinusitis and pulmonary infiltrates also occur in Churg-Strauss syndrome, primarily affecting the lung and heart. Peripheral neuropathy, coronary arteritis and gastrointestinal involvement are common.

Patients afflicted with Churg-Strauss syndrome can be diagnosed according to criteria established by the American College of Rheumatology (ACR). These criteria were intended to distinguish CSS from other forms of vasculitis. Not all patients meet every criterion. Some, in fact, may have only 2 or 3 criteria, yet they are still classified as Churg-Strauss syndrome. The ACR selected 6 disease features (criteria) as being those that best distinguished Churg-Strauss syndrome from other vasculitides. These criteria include: 1) asthma; 2) eosinophilia [ $>10\%$  on differential WBC count]; 3) mononeuropathy; 4) transient pulmonary infiltrates on chest X-rays; 5) paranasal sinus abnormalities; and 6) biopsy containing a blood vessel with extravascular eosinophils.

It is understood that all of the above-mentioned disorders include both the adult and juvenile forms of the disease where appropriate. It is also understood that all of the above-mentioned disorders include both chronic and acute forms of the disease wherein appropriate. In addition, the  $\text{TNF}\alpha$  antibody of the invention can be used to treat each of the above-mentioned  $\text{TNF}\alpha$ -related disorders alone or in combination with one another.

III. Pharmaceutical Compositions and Pharmaceutical Administration

A. Compositions and Administration

5           The antibodies, antibody-portions, and other TNF $\alpha$  inhibitors of the invention can be incorporated into pharmaceutical compositions suitable for administration to a subject who has vasculitis. Typically, the pharmaceutical composition comprises an antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable  
10 carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it will be preferable to include  
15 isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody, antibody portion, or other TNF $\alpha$  inhibitor.

20           The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the  
25 form of injectable or infusible solutions, such as compositions similar to those used for passive immunization of humans with other antibodies or other TNF $\alpha$  inhibitors. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the antibody or other TNF $\alpha$  inhibitor is administered by intravenous infusion or injection. In another preferred  
30 embodiment, the antibody or other TNF $\alpha$  inhibitor is administered by intramuscular or subcutaneous injection.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, antibody, antibody portion, or other TNF $\alpha$  inhibitor) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic agents. For example, an anti- hTNF $\alpha$  antibody or antibody portion of the invention may be coformulated and/or coadministered with one or more DMARD or one or more NSAID or one or more additional antibodies that bind other targets (*e.g.*, antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNF $\alpha$  receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNF $\alpha$  production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751) or any combination thereof. Furthermore, one or more antibodies of the invention may be used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible side effects, complications or low level of response by the patient associated with the various monotherapies.

In one embodiment, the invention pertains to pharmaceutical compositions comprising an effective amount of a TNF $\alpha$  inhibitor and a pharmaceutically acceptable carrier, wherein the effective amount of the TNF $\alpha$  inhibitor may be effective to treat vasculitides selected from the group consisting of group consisting of giant cell arteritis, 5 temporal arteritis, polymyalgia rheumatica, Takayasu's disease, polyarteritis nodosa, Kawasaki's disease, Behcet's Syndrome, Wegener's granulomatosis, and Churg-Strauss syndrome.

The antibodies, antibody-portions, and other TNF $\alpha$  inhibitors of the present invention can be administered by a variety of methods known in the art, although for many 10 therapeutic applications, the preferred route/mode of administration is intravenous injection or infusion. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, 15 transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, e.g., *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, 20 ed., Marcel Dekker, Inc., New York, 1978.

The TNF $\alpha$  antibodies of the invention can also be administered in the form of protein crystal formulations which include a combination of protein crystals encapsulated within a polymeric carrier to form coated particles. The coated particles of the protein crystal formulation may have a spherical morphology and be microspheres of up to 500 micro 25 meters in diameter or they may have some other morphology and be microparticulates. The enhanced concentration of protein crystals allows the antibody of the invention to be delivered subcutaneously. In one embodiment, the TNF $\alpha$  antibodies of the invention are delivered via a protein delivery system, wherein one or more of a protein crystal formulation or composition, is administered to a subject with a TNF $\alpha$ -related disorder. 30 Compositions and methods of preparing stabilized formulations of whole antibody crystals or antibody fragment crystals are also described in WO 02/072636, which is incorporated by reference herein. In one embodiment, a formulation comprising the crystallized

antibody fragments described in Examples 6 and 7 are used to treat a TNF $\alpha$ -related disorder.

In certain embodiments, an antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention may be orally administered, for example, with an inert diluent or an  
5 assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a  
10 compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

The pharmaceutical compositions of the invention may include a "therapeutically effective amount" or a "prophylactically effective amount" of an antibody or antibody  
15 portion of the invention. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody, antibody portion, or other TNF $\alpha$  inhibitor may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody, antibody portion, other TNF $\alpha$   
20 inhibitor to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody, antibody portion, or other TNF $\alpha$  inhibitor are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically,  
25 since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Dosage regimens may be adjusted to provide the optimum desired response (*e.g.*, a therapeutic or prophylactic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be  
30 proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used

herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are  
5 dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically  
10 effective amount of an antibody or antibody portion of the invention is 10-150 mg, more preferably 20-80 mg and most preferably about 40 mg. It is to be noted that dosage values may vary with the type and severity of the vasculitis. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person  
15 administering or supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The invention also pertains to packaged pharmaceutical compositions or kits which comprise a TNF $\alpha$  inhibitor of the invention and instructions for using the  
20 inhibitor to treat a particular disorder in which TNF $\alpha$  activity is detrimental, as described above. The package or kit alternatively can contain the TNF $\alpha$  inhibitor and it can be promoted for use, either within the package or through accompanying information, for the uses or treatment of the disorders described herein. The packaged pharmaceuticals or kits further can include a second agent (as described herein) packaged with or  
25 copromoted with instructions for using the second agent with a first agent (as described herein).

#### B. Additional therapeutic agents

The invention pertains to pharmaceutical compositions and methods of use  
30 thereof for the treatment of vasculitis, including Behcet's disease. The pharmaceutical compositions comprise a first agent that prevents or inhibits vasculitis. The pharmaceutical composition also may comprise a second agent that is an active



pharmaceutical ingredient; that is, the second agent is therapeutic and its function is beyond that of an inactive ingredient, such as a pharmaceutical carrier, preservative, diluent, or buffer. The second agent may be useful in treating or preventing vasculitis or another inflammatory disease. The second agent may diminish or treat at least one  
5 symptom(s) associated with the targeted disease. The first and second agents may exert their biological effects by similar or unrelated mechanisms of action; or either one or both of the first and second agents may exert their biological effects by a multiplicity of mechanisms of action. A pharmaceutical composition may also comprise a third compound, or even more yet, wherein the third (and fourth, etc.) compound has the same  
10 characteristics of a second agent.

It should be understood that the pharmaceutical compositions described herein may have the first and second, third, or additional agents in the same pharmaceutically acceptable carrier or in a different pharmaceutically acceptable carrier for each described embodiment. It further should be understood that the first, second, third and additional  
15 agent may be administered simultaneously or sequentially within described embodiments. Alternatively, a first and second agent may be administered simultaneously, and a third or additional agent may be administered before or after the first two agents.

The combination of agents used within the methods and pharmaceutical  
20 compositions described herein may have a therapeutic additive or synergistic effect on the condition(s) or disease(s) targeted for treatment. The combination of agents used within the methods or pharmaceutical compositions described herein also may reduce a detrimental effect associated with at least one of the agents when administered alone or without the other agent(s) of the particular pharmaceutical composition. For example,  
25 the toxicity of side effects of one agent may be attenuated by another agent of the composition, thus allowing a higher dosage, improving patient compliance, and improving therapeutic outcome. The additive or synergistic effects, benefits, and advantages of the compositions apply to classes of therapeutic agents, either structural or functional classes, or to individual compounds themselves.

30 Supplementary active compounds can also be incorporated into the compositions. In certain embodiments, an antibody or antibody portion of the invention is coformulated with and/or coadministered with one or more additional therapeutic

agents that are useful for treating inflammatory disorders in which TNF $\alpha$  activity is detrimental, including vasculitis. For example, an anti-hTNF $\alpha$  antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention may be coformulated and/or coadministered with one or more additional antibodies that bind other targets (*e.g.*,  
5 antibodies that bind other cytokines or that bind cell surface molecules), one or more cytokines, soluble TNF $\alpha$  receptor (see *e.g.*, PCT Publication No. WO 94/06476) and/or one or more chemical agents that inhibit hTNF $\alpha$  production or activity (such as cyclohexane-ylidene derivatives as described in PCT Publication No. WO 93/19751). Furthermore, one or more antibodies or other TNF $\alpha$  inhibitors of the invention may be  
10 used in combination with two or more of the foregoing therapeutic agents. Such combination therapies may advantageously utilize lower dosages of the administered therapeutic agents, thus avoiding possible toxicities or complications associated with the various monotherapies. Specific therapeutic agent(s) are generally selected based on the particular disorder being treated, as discussed below.

15 Nonlimiting examples of therapeutic agents with which an antibody, antibody portion, or other TNF $\alpha$  inhibitor of the invention can be combined include the following: non-steroidal anti-inflammatory drug(s) (NSAIDs); cytokine suppressive anti-inflammatory drug(s) (CSAIDs); CDP-571/BAY-10-3356 (humanized anti-TNF $\alpha$  antibody; Celltech/Bayer); cA2/infliximab (chimeric anti-TNF $\alpha$  antibody; Centocor); 75  
20 kdTNFR-IgG/etanercept (75 kD TNF receptor-IgG fusion protein; Immunex; see *e.g.*, *Arthritis & Rheumatism* (1994) Vol. 37, S295; *J. Invest. Med.* (1996) Vol. 44, 235A); 55 kdTNF-IgG (55 kD TNF receptor-IgG fusion protein; Hoffmann-LaRoche); IDEC-CE9.1/SB 210396 (non-depleting primatized anti-CD4 antibody; IDEC/SmithKline; see  
25 *e.g.*, *Arthritis & Rheumatism* (1995) Vol. 38, S185); DAB 486-IL-2 and/or DAB 389-IL-2 (IL-2 fusion proteins; Seragen; see *e.g.*, *Arthritis & Rheumatism* (1993) Vol. 36, 1223); Anti-Tac (humanized anti-IL-2R $\alpha$ ; Protein Design Labs/Roche); IL-4 (anti-inflammatory cytokine; DNAX/Schering); IL-10 (SCH 52000; recombinant IL-10, anti-inflammatory cytokine; DNAX/Schering); IL-4; IL-10 and/or IL-4 agonists (*e.g.*, agonist antibodies); IL-1RA (IL-1 receptor antagonist; Synergen/Amgen); TNF-bp/s-TNF  
30 (soluble TNF binding protein; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S284; *Amer. J. Physiol. - Heart and Circulatory Physiology* (1995) Vol.

268, pp. 37-42); R973401 (phosphodiesterase Type IV inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); MK-966 (COX-2 Inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S81); Iloprost (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S82); methotrexate;  
5 thalidomide (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282) and thalidomide-related drugs (*e.g.*, Celgen); leflunomide (anti-inflammatory and cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S131; *Inflammation Research* (1996) Vol. 45, pp. 103-107); tranexamic acid (inhibitor of plasminogen activation; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9  
10 (supplement), S284); T-614 (cytokine inhibitor; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); prostaglandin E1 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S282); Tenidap (non-steroidal anti-inflammatory drug; see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S280); Naproxen (non-steroidal anti-inflammatory drug; see *e.g.*, *Neuro Report* (1996) Vol. 7,  
15 pp. 1209-1213); Meloxicam (non-steroidal anti-inflammatory drug); Ibuprofen (non-steroidal anti-inflammatory drug); Piroxicam (non-steroidal anti-inflammatory drug); Diclofenac (non-steroidal anti-inflammatory drug); Indomethacin (non-steroidal anti-inflammatory drug); Sulfasalazine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S281); Azathioprine (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39,  
20 No. 9 (supplement), S281); ICE inhibitor (inhibitor of the enzyme interleukin-1 $\beta$  converting enzyme); zap-70 and/or lck inhibitor (inhibitor of the tyrosine kinase zap-70 or lck); VEGF inhibitor and/or VEGF-R inhibitor (inhibitors of vascular endothelial cell growth factor or vascular endothelial cell growth factor receptor; inhibitors of angiogenesis); corticosteroid anti-inflammatory drugs (*e.g.*, SB203580); TNF-convertase  
25 inhibitors; anti-IL-12 antibodies; anti-IL-18 antibodies; interleukin-11 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S296); interleukin-13 (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S308); interleukin-17 inhibitors (see *e.g.*, *Arthritis & Rheumatism* (1996) Vol. 39, No. 9 (supplement), S120); gold; penicillamine; chloroquine; hydroxychloroquine; chlorambucil;  
30 cyclophosphamide; cyclosporine; total lymphoid irradiation; anti-thymocyte globulin; anti-CD4 antibodies; CD5-toxins; orally-administered peptides and collagen; lobenzarit disodium; Cytokine Regulating Agents (CRAs) HP228 and HP466 (Houghten

Pharmaceuticals, Inc.); ICAM-1 antisense phosphorothioate oligodeoxynucleotides (ISIS 2302; Isis Pharmaceuticals, Inc.); soluble complement receptor 1 (TP10; T Cell Sciences, Inc.); prednisone; orgotein; glycosaminoglycan polysulphate; minocycline; anti-IL2R antibodies; marine and botanical lipids (fish and plant seed fatty acids; see 5 *e.g.*, DeLuca *et al.* (1995) *Rheum. Dis. Clin. North Am.* 21:759-777); auranofin; phenylbutazone; meclofenamic acid; flufenamic acid; intravenous immune globulin; zileuton; mycophenolic acid (RS-61443); tacrolimus (FK-506); sirolimus (rapamycin); amipribose (therafectin); cladribine (2-chlorodeoxyadenosine); azaribine; methotrexate; antivirals; and immune modulating agents. Any of the above-mentioned agents can be 10 administered in combination with the TNF $\alpha$  antibody of the invention to treat vasculitis, including Behcet's disease.

In one embodiment, the TNF $\alpha$  antibody of the invention is administered in combination with one of the following agents for the treatment of rheumatoid arthritis: methotrexate, prednisone, celecoxib, folic acid, hydroxychloroquine sulfate, rofecoxib, 15 etanercept, infliximab, leflunomide, naproxen, valdecoxib, sulfasalazine, methylprednisolone, ibuprofen, meloxicam, methylprednisolone acetate, gold sodium thiomalate, aspirin, azathioprine, triamcinolone acetonide, propoxyphene napsylate/apap, folate, nabumetone, diclofenac, piroxicam, etodolac, diclofenac sodium, oxaprozin, oxycodone hcl, hydrocodone bitartrate/apap, diclofenac sodium/misoprostol, fentanyl, 20 anakinra, human recombinant, tramadol hcl, salsalate, sulindac, cyanocobalamin/fa/pyridoxine, acetaminophen, alendronate sodium, prednisolone, morphine sulfate, lidocaine hydrochloride, indomethacin, glucosamine sulfate/chondroitin, cyclosporine, amitriptyline hcl, sulfadiazine, oxycodone hcl/acetaminophen, olopatadine hcl, misoprostol, naproxen sodium, omeprazole, 25 mycophenolate mofetil, cyclophosphamide, rituximab, IL-1 TRAP, MRA, CTLA4-IG, IL-18 BP, ABT-874, ABT-325 (anti-IL 18), anti-IL 15, BIRB-796, SCIO-469, VX-702, AMG-548, VX-740, Roflumilast, IC-485, CDC-801, and mesopram. In another embodiment, the TNF $\alpha$  antibody of the invention is administered for the treatment of a vasculitis in combination with one of the above mentioned agents for the treatment of 30 rheumatoid arthritis.

In one embodiment, the TNF $\alpha$  antibody of the invention is administered in combination with one of the following agents for the treatment of vasculitis in which

TNF $\alpha$  activity is detrimental: anti-IL12 antibody (ABT 874); anti-IL18 antibody (ABT 325); small molecule inhibitor of LCK; small molecule inhibitor of COT; anti-IL1 antibody; small molecule inhibitor of MK2; anti-CD19 antibody; and small molecule inhibitor of CXCR3.

5           In yet another embodiment, the TNF $\alpha$  antibody of the invention is administered in combination with an antibiotic or antiinfective agent. Antiinfective agents include those agents known in the art to treat viral, fungal, parasitic or bacterial infections. The term, "antibiotic," as used herein, refers to a chemical substance that inhibits the growth of, or kills, microorganisms. Encompassed by this term are antibiotic produced by a  
10 microorganism, as well as synthetic antibiotics (e.g., analogs) known in the art. Antibiotics include, but are not limited to, clarithromycin (Biaxin<sup>®</sup>), ciprofloxacin (Cipro<sup>®</sup>), and metronidazole (Flagyl<sup>®</sup>).

          In one embodiment, the TNF $\alpha$  antibody of the invention is administered in combination with an additional therapeutic agent in the treatment of Behcet's disease.  
15 Additional therapeutic agents which can be used to treat Behcet's disease include, but are not limited to, prednisone, cyclophosphamide (Cytoxan), Azathioprine (also called imuran, methotrexate, timethoprim/sulfamethoxazole (also called bactrim or septrin) and folic acid.

          Any one of the above-mentioned therapeutic agents, alone or in combination  
20 therewith, can be administered to a subject suffering from vasculitis in which TNF $\alpha$  is detrimental in combination with the TNF $\alpha$  antibody of the invention. In one embodiment, any one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered to a subject suffering from rheumatoid arthritis in addition to a TNF $\alpha$  antibody to treat vasculitis. In another embodiment, any  
25 one of the above-mentioned therapeutic agents, alone or in combination therewith, can be administered in combination with the TNF $\alpha$  antibody of the invention, to a subject suffering from an vasculitis, including Behcet's disease.

          This invention is further illustrated by the following examples which should not  
30 be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference

## **EXAMPLES**

### **Example 1: TNF $\alpha$ Inhibitor In Animal Model for Behcet's Disease**

5

#### *Study of TNF antibody in Behcet's syndrome mouse model*

The following study is performed using the mouse HSV model of Behcet's disease (Hirata, Y., *et al.* (1993) *Acta. Otolaryngol. Suppl.* 503:79). Earlobes of mice  
10 which express human TNF $\alpha$  (see *EMBO J* (1991) 10:4025-4031 for further description) are scratched with a needle, then inoculated with  $1.0 \times 10^6$  plaque-forming units (pfu)/mL of Herpes Simplex Virus type 1 (HSV1) (KOS strain) solution, which causes inflammatory cells to accumulate in and around the blood vessels. As a result, intestinal, oral, ear lobe, and genital epithelial lesions occur. A mouse with Behcet's disease-like  
15 syndrome is defined as a mouse with two or more symptoms, which are similar to the typical morphological changes seen in human Behcet's disease.

A monoclonal anti-TNF $\alpha$  antibody which is known to bind and neutralize mouse TNF $\alpha$ , *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) is  
20 administered to the HSV-induced Behcet's syndrome mice in a range of doses both before and after inoculation, or from the day of lesion occurrence. Appropriate placebo controls are also administered. Hair loss, ulceration of the mouth and genital skin, and eye involvement is monitored, and tissue samples are collected from lesions. Tissue samples are formalin-fixed and paraffin-embedded for sectional analysis. Lesion  
25 sections are stained with hematoxylin and eosin and examined for the appearance of inflammatory cell. As a control, 30 mice are inoculated at the same site with a culture medium. Four weeks later, a second inoculation is performed using the same method, followed by 16 weeks of observation.

Mice are examined for hair regrowth and a decrease in ulcerations in the treated  
30 mice as compared to placebo treated mice. Improvements in lesions in treated mice, as determined through visual inspection and histological analysis, noting a decrease in inflammation at the site of the ulceration and a decrease in the number of inflammatory

cells, *e.g.*, T cells, at the site of the ulceration also are further indications of improvements.

**Example 2: TNF $\alpha$  Inhibitor In Treating Kawasaki's Disease**

5

*Effect of TNF antibody in Kawasaki's Disease using L. casei mouse model*

Using the mouse *L. casei* model of Kawasaki's disease (Lehman, T.J., *et al.* (1985) *Arthritis Rheum* 28:652; Duong, T.T. (2002) *Int Immunol* 15:79; Brahn, E., *et al.* (1999) *Clin Immunol* 90:147), the following study is performed. Coronary arteritis is induced in mice expressing human TNF $\alpha$  (see above) with a single intraperitoneal (ip) injection of *Lactobacillus casei* cell fragments. It has been shown that histologic sections of the hearts of mice treated with *L. casei*, resemble the vasculitis and aneurysms observed in the medium-sized coronary arteries of children with Kawasaki disease (Lehman *et al.* (1985) *Arthritis Rheum* 28:652; Duong (2002) *Int Immunol* 15:79; Brahn *et al.* (1999) *Clin Immunol* 90:147).

*L. casei* injected mice are administered a either control placebo or a monoclonal anti-TNF $\alpha$  antibody which is known to bind and neutralize mouse TNF $\alpha$ , *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) intraperitoneally through standard protocols. Hearts from injected mice are harvested on day 14 (early disease) or at the end of the study (established disease). Histologic sections are scored blindly for vasculitis.

A decrease in coronary arteritis is assessed by determining a reduction in inflammatory lesions of the coronary vessel wall of TNF antibody treated mice as compared to placebo treated animals. A decrease in coronary arteritis is assessed as a reduction in inflammatory mononuclear cell infiltrate of the coronary vessel wall accompanied by a reduction in intimal proliferation and less narrowing of the vessel lumen as compared to placebo treated animals.

30

**Example 3: TNF $\alpha$  Inhibitor In Animal Model for Kawasaki's Disease**

*TNF antibody in Kawasaki's Disease using ANCA Mouse Model*

The following study is performed using the mouse anti-endothelial cell antibodies (ANCA) model of Kawasaki's Disease (Grunebaum *et al.* (2002) *Clin. Exp. Immunol.* 130:233; Blank *et al.* (1995) *Clin. Exp. Immunol.* 102:120; Tomer *et al.* (1995) *Arthritis Rheum.* 38:1375; Damianovich *et al.* (1996) *J. Immunol.* 156:4946). Animals are immunized with anti-endothelial cell antibodies (ANCA) containing proteinase 3-specific antibodies derived from a Wegener's granulomatosis patient's plasma. Mice are immunized with purified ANCA and control mice are injected with normal IgG. Mice are administered weekly doses of either a placebo or a monoclonal anti-TNF $\alpha$  antibody which is known to bind and neutralize mouse TNF $\alpha$ , *e.g.*, antibody TN3 (TN3-19.12) (see Marzi *et al.* (1995) *Shock* 3:27; Williams *et al.* (1992) *Proc Natl Acad Sci U S A.* 89:9784; BD Biosciences Pharmingen) for upto four months. Three months after the immunization with the human ANCA, mice develop endogenous antibodies to ANCA. Mice are euthanized, and lungs, kidneys, and heart are examined histologically for lymphoid cell infiltration surrounding arterioles and venules, as well as deposition of Igs at the outer part of blood vessel walls like that observed in patients with Kawasaki's Disease (Grunebaum *et al.* (2002) *Clin. Exp. Immunol.* 130:233; Blank *et al.* (1995) *Clin. Exp. Immunol.* 102:120; Tomer *et al.* (1995) *Arthritis Rheum.* 38:1375; Damianovich *et al.* (1996) *J. Immunol.* 156:4946). A decrease in lymphoid cell infiltration and IgG deposition in vessel walls and a decrease in antibody titre of ANCA is indicative of an improvement in Kawasaki's disease.

**Example 4: TNF $\alpha$  Inhibitor in Treatment of Kawasaki's Disease**

*Clinical study of D2E7 in human subjects with Kawasaki's disease*

Patients suffering from Kawasaki's Disease (KD) are enrolled into the study; all patients have fever and at least 4 of the 5 clinical criteria published for KD (Barron, K.S., *et al.* (1999) *J. Rheumatol.* 26:170). Case-controls are also identified. The diameter of the coronary arteries is measured by echocardiography and corrected for body surface area. Electrocardiograms are screened for typical changes that may be



present in KD including prolonged PR or QT interval, abnormal Q waves, ST- and T-wave changes, low voltages or arrhythmias. KD patients are administered either D2E7 in biweekly and weekly doses of 40 mg or a placebo. Dosages may be adjusted by an ordinarily skilled artisan knowledgeable in KD. Patients are monitored for fever  
5 reduction. Adjuvant therapy, e.g. corticosteroids, are administered as needed. Patients are monitored and follow-up echocardiography is used to determine if coronary artery damage has occurred or whether an improvement in coronary artery lesions, demonstrated through improved echocardiogram results has occurred.

10 **Example 5: TNF $\alpha$  Inhibitor in Treatment of Behcet's Disease**

*Clinical Study of D2E7 in Human Subjects With Behcet's Disease*

Patients for the study are selected because they fulfill International Study Group  
15 criteria, which requires the presence of oral ulceration plus any two of genital ulceration, typical defined eye lesions, typical defined skin lesions, or a positive pathergy test (*Lancet*. (1990) 335:1078; Kaklamani, V.G. *et al.* (2001) *Semin. Arthritis Rheum.* 30:299) for a mean of 6 years. Behcet's patients are administered either D2E7 in biweekly and weekly doses of 40 mg or a placebo. Dosages may be adjusted by an  
20 ordinarily skilled artisan knowledgeable in Behcet's disease. Treated and placebo patients are given a systemic examination and detailed ophthalmological assessment, including visual acuity, measurement of intraocular pressure, slit-lamp biomicroscopy, and indirect ophthalmoscopy of the posterior segment followed by fundus photography, both before and following the treatment regime. Patients are examined for an  
25 improvement in the documented symptoms associated with Behcet's disease, e.g., reduction in eye inflammation and reduction in number or severity of mouth ulcers.

**Example 6: Crystallization of D2E7 F(ab)'<sub>2</sub> fragment**

30 *Generation and purification of the D2E7 F(ab)'<sub>2</sub> Fragment*

A D2E7 F(ab)'<sub>2</sub> fragment was generated and purified according to the following procedure. Two ml of D2E7 IgG (approximately 63 mg/ml) was dialyzed against 1 liter

of Buffer A (20 mM NaOAc, pH 4) overnight. After dialysis, the protein was diluted to a concentration of 20 mg/ml. Immobilized pepsin (Pierce; 6.7 ml of slurry) was mixed with 27 ml of Buffer A, mixed, and centrifuged (Beckman floor centrifuge, 5000 rpm, 10 min). The supernatant was removed, and this washing procedure was repeated twice  
5 more. The washed immobilized pepsin was re-suspended in 13.3 ml of Buffer A. D2E7 (7.275 ml, 20 mg/ml, 145.5 mg) was mixed with 7.725 ml of Buffer A and 7.5 ml of the washed immobilized pepsin slurry. The D2E7/pepsin mixture was incubated at 37 °C for 4.5 hr with shaking (300 rpm). The immobilized pepsin was then separated by centrifugation. Analysis of the supernatant by SDS-PAGE indicated that the digestion of  
10 D2E7 was essentially complete (~115 kDa band unreduced, ~30 and ~32 kDa bands reduced).

The D2E7 F(ab)<sub>2</sub> fragment was separated from intact D2E7 and Fc fragments using Protein A chromatography. One-half of the above reaction supernatant (10 ml) was diluted with 10 ml of Buffer B (20 mM Na phosphate, pH 7), filtered through a 0.45  
15 µm Acrodisc filter, and loaded onto a 5 ml Protein A Sepharose column (Pharmacia Hi-Trap; previously washed with 50 ml of Buffer B). Fractions were collected. After the protein mixture was loaded, the column was washed with Buffer B until the absorbance at 280 nm re-established a baseline. Bound proteins were eluted with 5 ml of Buffer C (100 mM citric acid, pH 3); these fractions were neutralized by adding 0.2 ml of 2 M  
20 Tris•HCl, pH 8.9. Fractions were analyzed by SDS-PAGE; those that contained the D2E7 F(ab)<sub>2</sub> fragment were pooled (~42 ml). Protein concentrations were determined by absorbance at 280 nm in 6 M guanidine•HCl, pH 7 (calculated extinction coefficients: D2E7, 1.39 (AU-ml)/mg; F(ab)<sub>2</sub>, 1.36 (AU-ml)/mg). The flow-through pool contained ~38.2 mg protein (concentration, 0.91 mg/ml), which represents a 79% yield  
25 of F(ab)<sub>2</sub> (theoretical yield is ~2/3 of starting material, divided by two [only half purified], i.e. ~48.5 mg).

The D2E7 F(ab)<sub>2</sub> fragment was further purified by size-exclusion chromatography. The pooled Protein A flow-through was concentrated from ~42 to ~20 ml, and a portion (5 ml, ~7.5 mg) was then chromatographed on a Superdex 200 column  
30 (26/60, Pharmacia) previously equilibrated (and eluted) with Buffer D (20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA). Two peaks were noted by absorbance at 280 nm: Peak 1, eluting at 172–200 ml, consisted of F(ab)<sub>2</sub> (analysis by SDS-PAGE; ~115 kDa

band unreduced, ~30 and ~32 kDa bands reduced); Peak 2, eluting at 236–248 ml, consisted of low molecular weight fragment(s) (~15 kDa, reduced or unreduced). Peak 1 was concentrated to 5.3 mg/ml for crystallization trials.

### 5 Crystallization of the D2E7 F(ab)<sub>2</sub> Fragment

The D2E7 F(ab)<sub>2</sub> fragment (5.3 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method by mixing equal volumes of F(ab)<sub>2</sub> and crystallization buffer (approx. 1 µl of each) and allowing the mixture to equilibrate against the crystallization Buffer Bt 4 or 18 °C. The crystallization buffers used consisted of the Hampton Research Crystal Screens I (solutions 1–48) and II (solutions 1–48), Emerald Biostructures Wizard Screens I and II (each solutions 1–48), and the Jena Biosciences screens 1–10 (each solutions 1–24). Crystals were obtained under many different conditions, as summarized in Table 1.

Table 1. Summary of crystallization conditions for the D2E7 F(ab)<sub>2</sub> fragment.

15

Screen	Solution	Temp °C	Condition	Result
Hampton 1	32	4	2.0 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
Hampton 1	46	4	0.2 M Ca(Oac) <sub>2</sub> , 0.1 M Na cacodylate pH 6.5, 18% PEG 8K	medium sized needle clusters
Hampton 1	48	4	0.1 M Tris HCl pH 8.5, 2.0 M NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub>	micro needle clusters
Hampton 2	2	4	0.01 M hexadecyltrimethylammonium bromide, 0.5 M NaCl, 0.01 M MgCl <sub>2</sub>	small shard crystals
Hampton 2	13	4	0.2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M NaOAc pH 4.6, 30% PEG MME 2000	small needle clusters
Hampton 2	15	4	0.5 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 5.6, 1.0M Li <sub>2</sub> SO <sub>4</sub>	large needle clusters
Hampton 2	16	4	0.5M NaCl, 0.1M NaOAc pH 5.6, 4% Ethylene Imine polymer	large irregular crystal
Hampton 1	34	18	0.1 NaOAc pH 4.6, 2.0 M Na Formate	needle clusters
Hampton 1	35	18	0.1M Hepes pH 7.5, 0.8M mono-sodium dihydrogen phosphate, 0.8M mono-potassium dihydrogen phosphate	needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2.0M NaCl	dense needle clusters
Hampton 2	12	18	0.1M CdCl <sub>2</sub> , 0.1M NaOAc pH 4.6, 30% PEG 400	needles & amorphous crystals
Hampton 2	15	18	0.5M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 5.6, 1.0M Li <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
Wizard I	27	4	1.2M NaH <sub>2</sub> PO <sub>4</sub> , 0.8M K <sub>2</sub> HPO <sub>4</sub> , 0.1M CAPS pH 10.5, 0.2 M Li <sub>2</sub> SO <sub>4</sub>	Medium large needle clusters
Wizard I	30	4	1.26M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1 M NaOAc pH 4.5, 0.2M NaCl	small needle clusters
Wizard II	8	4	10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl	Large plate crystals grown in clusters

Screen	Solution	Temp °C	Condition	Result
Wizard II	43	4	10% PEK 8K, 0.1M Tris pH 7.0, 0.2 M MgCl <sub>2</sub>	micro needle clusters
Wizard I	4	18	35% MPD, 0.1M Imidazole pH 8.0, 0.2M MgCl <sub>2</sub>	rod shaped crystal
Wizard I	27	18	1.2M NaH <sub>2</sub> PO <sub>4</sub> , 0.8M K <sub>2</sub> HPO <sub>4</sub> , 0.1M CAPS pH 10.5, 0.2 M Li <sub>2</sub> SO <sub>4</sub>	Needle clusters
Wizard II	7	18	30% PEG 3K, 0.1M Tris pH 8.5, 0.2M NaCl	tiny needle clusters
Wizard II	11	18	10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac) <sub>2</sub>	tiny hexagonal or rhombohedral crystals
Wizard II	46	18	1.0M AP, 0.1M Imidazole pH 8.0, 0.2M NaCl	1 irregular crystal
JB 1	D6	4	30% PEG 3K, 0.1M Tris HCl pH 8.5, 0.2M Li <sub>2</sub> SO <sub>4</sub>	tiny needles in precipitate
JB 2	B6	4	20% PEG 4K, 0.1M Tris HCl pH 8.5, 0.2M Na Cacodylate	tiny needle cluster balls
JB 3	A1	4	8% PEG 4K, 0.8M LiCl, 0.1M Tris HCl pH 8.5	Large frost-like crystals
JB 3	B1	4	15% PEG 4K, 0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
JB 3	D5	4	30% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH <sub>4</sub> OAc	tiny needles in precipitate.
JB 4	B1	4	15% PEG 6K, 0.05M KCl, 0.01M MgCl <sub>2</sub>	needle cluster balls
JB 3	A6	18	12% PEG 4K, 0.1M NaOAc pH 4.6, 0.2M NH <sub>4</sub> OAc	needle clusters
JB 3	B1	18	15% PEG 4K, 0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	needle clusters in precipitate
JB 3	C6	18	25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M NH <sub>4</sub> OAc	long, thin needles
JB 4	C5	18	8% PEG 8K, 0.2 M LiCl, 0.05M MgSO <sub>4</sub>	frost-like crystals
JB 5	A3	4	15% PEG 8K, 0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	long single needles in phase separation
JB 5	A4	4	15% PEG 8K, 0.5M Li <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
JB 5	A5	4	15% PEG 8K, 0.1M Na MES pH 6.5, 0.2M Ca(OAc) <sub>2</sub>	needle cluster balls
JB 6	B2	4	1.6M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.5 LiCl	tiny needle cluster balls
JB 6	C2	4	2.0 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 4.6	micro needle clusters
JB 10	D3	18	2.0M Na Formate, 0.1M NaOAc pH 4.6	needle clusters

The following conditions (as described in Table 1) produced crystals which can be used for diffraction quality crystals: Wizard II, 11, 18, 10% 2-propanol, 0.1M cacodylate pH 6.5, 0.2M Zn(Oac)<sub>2</sub>, tiny hexagonal or rhom. Xtals; Wizard II, 10% PEG 8K, 0.1M Na/K phosphate pH 6.2, 0.2M NaCl, large plate xtals grown in clusters; JB 3, C6, 18, 25% PEG 4K, 0.1M Na Citrate pH 5.6, 0.2M Ammonium Acetate, long, thin needles; Hampton 2, 15, 18, 0.5M AS, 0.1M Na Acetate trihydrate pH 5.6, 1.0M Li Sulfate monohydrate, tiny needle clusters.

#### 10 **Example 7: Crystallization of D2E7 Fab fragment**

*Generation and purification of the D2E7 Fab Fragment*

A D2E7 Fab fragment was generated and purified according to the following procedure. Four ml of D2E7 IgG (diluted to about 20 mg/ml) was diluted with 4 ml of Buffer E (20 mM Na phosphate, 5 mM cysteine•HCl, 10 mM EDTA, pH7) and mixed with 6.5 ml of a slurry of immobilized papain (Pierce, 1%; previously washed twice with 26 ml of Buffer E). The D2E7/papain mixture was incubated at 37 °C overnight with shaking (300 rpm). The immobilized papain and precipitated protein were separated by centrifugation; analysis of the supernatant by SDS-PAGE indicated that the digestion of D2E7 was partially complete (~55, 50, 34, and 30 kDa bands unreduced, with some intact and partially digested D2E7 at ~115 and ~150 kDa; ~30 and ~32 kDa bands reduced, as well as a ~50 kDa band). Nonetheless, the digestion was halted and subjected to purification.

The D2E7 Fab fragment was purified by Protein A chromatography and Superdex 200 size-exclusion chromatography essentially as described above for the F(ab)<sub>2</sub> fragment. The Protein A column flow-through pool (21 ml) contained ~9.2 mg (0.44 mg/ml), whereas the Protein A eluate (4 ml) contained ~19.5 mg (4.9 mg/ml). Analysis by SDS-PAGE indicated that the flow-through was essentially pure Fab fragment (~48 and ~30 kDa unreduced, broad band at ~30 kDa reduced), whereas the eluate was intact and partially-digested D2E7. The Fab fragment was further purified on a Superdex 200 column, eluting at 216–232 ml, i.e., as expected, after the F(ab)<sub>2</sub> fragment but before the small Fc fragments. The D2E7 Fab fragment concentrated to 12.7 mg/ml for crystallization trials, as described below.

#### ***Crystallization of the D2E7 Fab Fragment***

The D2E7 Fab fragment (12.7 mg/ml in 20 mM HEPES, pH 7, 150 mM NaCl, 0.1 mM EDTA) was crystallized using the sitting drop vapor diffusion method essentially as described above for the F(ab)<sub>2</sub> fragment. Crystals were obtained under many different conditions, as summarized in **Table 2**.

Table 2. Summary of crystallization conditions for the D2E7 Fab fragment.

Screen	Solution	Temp °C	Condition	Result
Hampton 1	4	4	0.1M Tris pH 8.5, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	wispy needles
Hampton 1	10	4	0.2M NH <sub>4</sub> OAc, 0.1M NaOAc pH 4.6, 30% PEG 4K	wispy needle clusters

Screen	Solution	Temp °C	Condition	Result
Hampton 1	18	4	0.2M Mg(OAc) <sub>2</sub> , 0.1M Na Cacodylate pH 6.5, 20% PEG 8K	needle clusters
Hampton 1	20	4	0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 4.6, 25% PEG 4K	tiny needle clusters
Hampton 1	32	4	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	long, wispy needles
Hampton 1	33	4	4M Na Formate	tiny needle clusters
Hampton 1	38	4	0.1M Hepes pH 7.5	tiny needle clusters
Hampton 1	43	4	30% PEG 1500	tiny needle clusters
Hampton 1	46	4	0.2M Ca(OAc) <sub>2</sub> , 0.1M Na Cacodylate pH 6.5, 18% PEG 8K	large plate clusters
Hampton 1	47	4	0.1M NaOAc pH 4.6, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	long, wispy needles
Hampton 2	1	4	2M NaCl, 10% PEG 6K	small plate clusters
Hampton 2	2	4	0.01M Hexadecyltrimethylammonium bromide, 0.5M NaCl, 0.01 MgCl <sub>2</sub>	round & irregular plates
Hampton 2	5	4	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 5% isopropanol	long fiber ropes
Hampton 2	13	4	0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny, wispy needle clusters
Hampton 2	14	4	0.2M K/Na Tatrte, 0.1M Na Citrate pH 5.6, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
Hampton 2	27	4	0.01M ZnSO <sub>4</sub> , 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Hampton 2	28	4	30% MPD	tiny needle clusters
Hampton 1	4	18	0.1M Tris pH 8.5, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	needle clusters
Hampton 1	9	18	0.2M NH <sub>4</sub> OAc, 0.1M Na Citrate pH 5.6, 30% PEG 4K	needle clusters
Hampton 1	17	18	0.2M Li <sub>2</sub> SO <sub>4</sub> , 0.1M Tris pH 8.5, 30% PEG 4K	long, wispy needles
Hampton 1	32	18	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	needle clusters
Hampton 1	33	18	4M Na Formate	tiny needle clusters
Hampton 1	38	18	0.1M Hepes pH 7.5	fiber bundles
Hampton 1	43	18	30% PEG 1500	tiny needle clusters
Hampton 1	47	18	0.1M NaOAc pH 4.6, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	tiny needle clusters
Hampton 2	1	18	2M NaCl, 10% PEG 6K	long, wispy needle clusters
Hampton 2	5	18	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 5% 2-propanol	tiny needle clusters
Hampton 2	9	18	0.1M NaOAc pH 4.6, 2M NaCl	long, wispy needles
Hampton 2	13	18	0.2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 4.6, 25% PEG MME 2K	tiny needle clusters
Hampton 2	14	18	0.2M K/Na Tartrate, 0.1M Na Citrate pH 5.6, 2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	long wispy needles
Hampton 2	27	18	0.01M ZnSO <sub>4</sub> , 0.1 MES pH 6.5, 25% PEG MME 550	tiny needle clusters
Wizard I	20	4	0.4M NaH <sub>2</sub> PO <sub>4</sub> /1.6M K <sub>2</sub> HPO <sub>4</sub> , 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	28	4	20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl	large orthorhombic plate clusters
Wizard I	31	4	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	wispy needle clusters
Wizard I	39	4	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li <sub>2</sub> SO <sub>4</sub>	needle clusters
Wizard II	3	4	20% PEG 8K, 0.1M Tris pH 8.5, 0.2M MgCl <sub>2</sub>	large hexagonal or orthorhombic plate cluster in phase sep

Screen	Solution	Temp °C	Condition	Result
Wizard II	4	4	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M Cacodylate pH 6.5, 0.2 NaCl	tiny needle clusters
Wizard II	9	4	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M phosphate citrate pH 4.2	tiny, wispy needle clusters
Wizard II	28	4	20% PEG 8K, 0.1M MES pH 6, 0.2M Ca(OAc) <sub>2</sub>	tiny needle clusters; large wispy needle clusters
Wizard II	35	4	0.8M NaH <sub>2</sub> PO <sub>4</sub> /1.2M K <sub>2</sub> HPO <sub>4</sub> , 0.1M NaOAc pH 4.5	tiny fiber bundles
Wizard II	38	4	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li <sub>2</sub> SO <sub>4</sub>	long wispy needles
Wizard II	47	4	2.5M NaCl, 0.1M Imidazole pH 8, 0.2M Zn(OAc) <sub>2</sub>	tiny needle clusters
Wizard I	6	18	20% PEG 3K, 0.1M Citrate pH 5.5	needle clusters
Wizard I	20	18	0.4M NaH <sub>2</sub> PO <sub>4</sub> /1.6M K <sub>2</sub> HPO <sub>4</sub> , 0.1M Imidazole pH 8, 0.2M NaCl	tiny needle clusters
Wizard I	27	18	1.2M NaH <sub>2</sub> PO <sub>4</sub> /0.8M K <sub>2</sub> HPO <sub>4</sub> , 0.1M CAPS pH 10, 0.2M Li <sub>2</sub> SO <sub>4</sub>	wispy needle clusters
Wizard I	30	18	1.26M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M NaOAc pH 4.5, 0.2M NaCl	wispy needles
Wizard I	31	18	20% PEG 8K, 0.1M phosphate citrate pH 4.2, 0.2M NaCl	tiny needle clusters
Wizard I	33	18	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M CAPS pH 10.5, 0.2M Li <sub>2</sub> SO <sub>4</sub>	fiber bundles
Wizard I	39	18	20% PEG 1K, 0.1M phosphate citrate pH 4.2, 0.2M Li <sub>2</sub> SO <sub>4</sub>	needle clusters
Wizard II	4	18	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M Cacodylate pH 6.5, 0.2 NaCl	needle clusters
Wizard II	9	18	2M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 0.1M phosphate citrate pH 4.2	wispy needles
Wizard II	35	18	0.8M NaH <sub>2</sub> PO <sub>4</sub> /1.2M K <sub>2</sub> HPO <sub>4</sub> , 0.1M NaOAc pH 4.5	tiny needle clusters
Wizard II	38	18	2.5M NaCl, 0.1M NaOAc pH 4.5, 0.2M Li <sub>2</sub> SO <sub>4</sub>	tiny needle clusters

The following conditions (as described in Table 2) produced crystals which can be used for diffraction quality crystals: Hampton 2, 1, 4C, 2M NaCl, 10% PEG 6K; small plate clusters; Hampton 1 46, 4C, 0.2M Ca Acetate, 0.1M Na Cacodylate, pH 6.5, 18% PEG 8K, large plate clusters; Wizard I, 28, 4C, 20% PEG 3K, 0.1M Hepes pH 7.5, 0.2M NaCl, large orthorhombic plate clusters; Wizard II 3, 4C, 20% PEG 8K, 0.1M Tris pH 8.5, 0.2M MgCl<sub>2</sub>, lrg hex or orth plate cluster in phase sep.

### EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.